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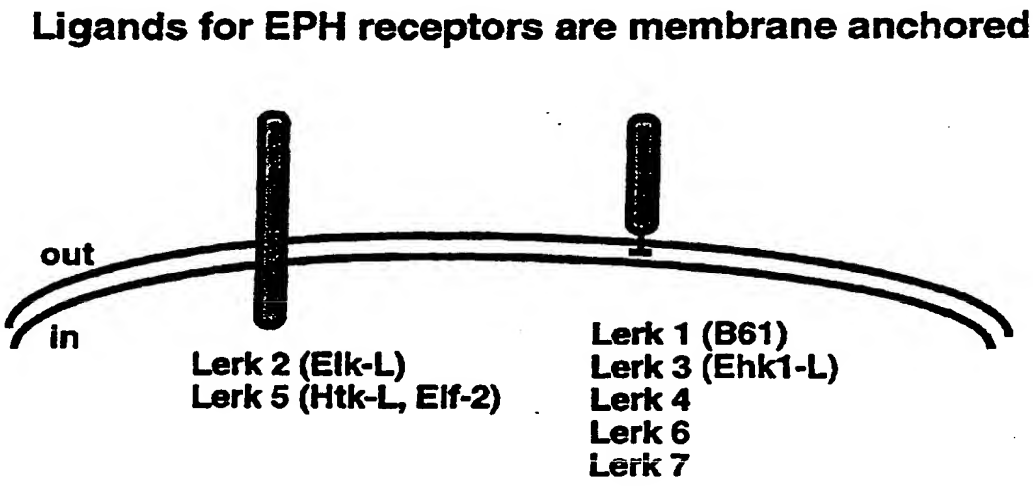

(57) Abstract

A novel ligand regulatory pathway is disclosed and methods of activating the novel pathway in a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase protein. Methods are provided for identifying substances capable of activating the ligand regulatory pathway. Therapeutic methods for affecting neuronal development and regeneration and pharmaceutical compositions using the substances and Eph subfamily receptor tyrosine kinase proteins are also described.

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Title: Method of Activating a Novel Ligand Regulatory Pathway

FIELD OF THE INVENTION

The invention relates to a novel ligand regulatory pathway, to methods for identifying substances capable of activating the novel pathway, methods for assaying for agonists or antagonists of the novel pathway, and to methods and pharmaceutical compositions for affecting neuronal development and regeneration.

BACKGROUND OF THE INVENTION

Embryonic development of multicellular organisms is a highly ordered process that requires coordination of individual cells. Every cell must decipher the numerous signals it receives and then properly execute commands in order to achieve the correct position and differentiated state in the animal. The exquisite controls over cell growth, determination, migration and adhesion are mediated by molecules located on the plasma membrane surface.

A class of membrane associated molecules known to regulate cellular interactions are receptor tyrosine kinase proteins. The evolutionary conservation of genes encoding receptor tyrosine kinase proteins and their targets has emphasized the importance of these proteins in intracellular communication, and has also provided model systems for genetic analysis of tyrosine kinase signalling pathways.

A growing number of closely related transmembrane receptor tyrosine kinase proteins containing cell adhesion-like domains on their extracelluar surface have been identified. Collectively, this group of proteins defines the *Eph* subfamily, which is made up of at least thirteen related but unique gene sequences in higher vertebrates (Hirai *et al.*, *Science* 238:1717-1720, 1987; Letwin *et al.*, *Oncogene* 3:621-627, 1988; Lindberg *et al.*, *Mol. Cell. Biol.* 10:6316-6324, 1990; Lhotak *et al.*, *Mol. Cell. Biol.* 11:2496-2502, 1991; Chan and Watt, *Oncogene* 6:1057-1061, 1991; Lai and Lemke, *Neuron* 6:691-704, 1991; Pasquale, *Cell Regulation* 2:523-534, 1991; Sajjadi *et al.*, *New Biologist* 3:769-778, 1991; Wicks *et al.*, *PNAS* 89:1611-1615, 1992; Gilardi-Hebenstreit *et al.*, *Oncogene* 7:2499-2506, 1992; Bohme *et al.*, *Oncogene* 8:2857-2862, 1993; Sajjadi and Pasquale, *Oncogene* 8:1801-1813, 1993). The presence of cell adhesion-like domains in this family of tyrosine kinases suggests that these proteins function in cell-cell interactions.

The other major families of proteins implicated in cell adhesion include the cadherins, selectins, integrins, and those of the immunoglobulin superfamily (reviewed by Hynes, R.O. and Landers, A.D., Cell 68, 303-322, 1992). The extracelluar regions of cell adhesion molecules frequently contain peptide repeats, such as FN III motifs, epidermal growth factor (EGF) repeats, or Ig loops that may direct protein-protein interactions at the cell surface. A number of cell adhesion molecules in both vertebrates (Dodd, J. and Jessell, T.M., Science, 242, 692-699, 1988; Jessell, T.M., Neuron, 1, 3-13, 1988; Furley *et al.*, Cell 61, 157-170, 1990; Burns *et al.*, Neuron, 7, 209-220, 1991) and invertebrates (Bastiani et al., *Cell* 48:745-755, 1987; Elkins *et al.*, *Cell* 60:565-575, 1990; Grenningloh *et al.*, *Cold Spring Harb.*

Symp. Quant. Biol. 55, 327-340, 1991; Nose *et al., Cell* 70:553-567, 1992) have been implicated in axonal growth cone guidance and pathway/target recognition. Other aspects of neuronal morphogenesis involving cell-cell interactions may also require the activities of cell adhesion molecules (Edelman and Thiery, In *The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants*, Wiley, New York, 1985; Hatta *et al., Dev. Biol.* 120:215-227, 1987; Takeichi, *Development* 102:639-655, 1988; Takeichi, *Annu. Rev. Biochem.* 59:237-252 1990; Takeichi, *Science* 251:1451-1455, 1991; Edelman, *Biochemistry* 27:3533-3543, 1988; Grumet, *Curr. Opin. Neurobiol.* 1:370-376, 1991; Hynes and Lander, *Cell* 68:303-322, 1992). For example, ectopic N-cadherin expression during gastrulation stage *Xenopus* embryos has been shown to interfere with segregation of the neural tube from the ectoderm (Detrick *et al., Neuron* 4:493-506, 1990; Fujimori *et al., Development* 110:97-104, 1990). Although many different types of cell adhesion molecules have been identified, little is known about how these adhesive interactions are regulated and how they function in cell signalling pathways during normal development.

A critical stage in the development of the nervous system is the projection of axons to their targets. Navigational decisions are made at the growth cones of the migrating axons. As axons grow their growth cones extend and retract filopodia and lamellipodia processes which are implicated in the navigational decisions and pathfinding abilities of migrating axons. Like peripheral nervous system axons, the growth cones of neurons associated with the central nervous system follow stereotyped pathways and apparently can selectively chose from a number of possible routes (reviewed by Goodman and Shatz, *Cell* 72:77-98, 1993). Early pathways in the vertebrate embryonic brain are thought to be arranged as a set of longitudinal tracts connected by commissures. However, the molecular mechanisms that underlay growth cone navigation axon pathfinding and commissure formation in development are poorly understood (Hynes, R.O. and Lander, A.D., 1992, Cell 68:303).

It is a fundamental principle of nervous system wiring that the projections of neurons from one region of the nervous system to another are organized topographically. During embryonic development a multitude of incoming axons must find and connect with a corresponding set of target cells to form a continuous topographic map. It has been suggested that formation and refinement of the topographic map of neurons may be directed in part by positional labels displayed on the surface of developing and migrating neurons. However, to date such positional labels have not been identified (Tessier-Lavigne, 1995, *Cell* 82:345-348). Recently, ligands for receptor tyrosine kinases of the Eph subfamily have been implicated as positional labels in the retinotectal system (Drescher et al., 1995 *Cell* 82:359-370).

The developmental function of tyrosine kinases during axonogenesis has been studied in Drosophila. A function in axonal pathfinding is evident for the Drosophila abl tyrosine kinase when *abl* mutations are combined with mutations in other genes including the neural cell adhesion molecule, *fasciclin* I (*fas* I, Elkins *et al., Cell* 60:565-575, 1990) or

disabled (*dab*, Gertler *et al.*, *Cell* 58:103-113, 1989). Thes studies have shown that the abl tyrosine kinase is specifically localized to the axonal compartment of the embryonic Central Nervous System (CNS) (Gertler *et al.*, *Cell* 58:103-113, 1989). Moreover, genetic analysis has indicated that subcellular localization to axons is essential for abl function during development (Henkemeyer *et al.*, *Cell* 63:949-960, 1990) and that mutations in second-site modifier genes including *fas* I and *dab* can reveal a role for abl in axonogenesis (Elkins *et al.*, *Cell* 60:565-575, 1990; Gertler *et al.*, *Cell* 58:103-113 1989). The requirement for tyrosine phosphorylation in axonal outgrowth and adhesion in Drosophila is strengthened by the identification in CNS axons of three transmembrane tyrosine phosphatases containing FN III motifs (Tian *et al.*, *Cell* 67:675-685, 1991; Yang *et al.*, *Cell* 67:661-673, 1991).

SUMMARY OF THE INVENTION

The present inventors have identified and characterized a novel ligand regulatory pathway that plays a crucial role in cell-cell interactions and axonogenesis in the development and regeneration of the nervous system. The present inventors have determined that Eph subfamily receptor tyrosine kinases activate a ligand regulatory pathway in cells expressing ligands for the Eph subfamily receptor tyrosine kinases. Activation of the ligand regulatory pathway results in downstream activation of a series of regulatory pathways in the cells that control gene expression, cell division, cytoskeletal architecture, cell metabolism, cell migration and cell-cell interactions. The ligand regulatory pathway may be activated by an Eph subfamily receptor tyrosine kinase lacking in an active catalytic kinase domain.

In particular, the inventors have demonstrated that expression of an Eph subfamily receptor tyrosine kinase is essential for formation of a commissure in the brain and that this essential function is independent of an intact catalytic kinase domain. The direct demonstration of a vital function in neuronal development for an Eph subfamily receptor tyrosine kinase is unprecedented, as is the showing of a function for a receptor tyrosine kinase which is mediated by the extracellular domain, independently of the catalytic kinase domain of the receptor. The inventors have demonstrated for the first time that a protein having the extracellular, transmembrane and juxtamembrane domains of an Eph subfamily receptor tyrosine kinase can provide a signal to a cell expressing a ligand for the receptor tyrosine kinases and thereby activate a ligand regulatory pathway in the cell expressing the ligand.

Accordingly, the present invention provides a method of activating a ligand regulatory pathway in a cell, comprising reacting an Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein, with a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the surface of the cell thereby activating the ligand regulatory pathway in the cell. In an embodiment, the protein or part of the protein is lacking in catalytic kinase activity. In a

further embodiment, the part of the protein comprises an extracellular, transmembrane and juxtamembrane domain, or only an extracellular domain of an Eph subfamily receptor tyrosine kinase, preferably Nuk.

The invention also provides a method for identifying a substance which is capable of binding to a ligand for an Eph subfamily receptor tyrosine kinase and activating a ligand regulatory pathway in a cell, comprising reacting a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the surface of the cell, with at least one test substance, under conditions which permit the formation of substance-ligand complexes, and assaying for substance-ligand complexes, for free substance, for non-complexed ligands, or for activation of the ligand.

Activation of the ligand may be assayed by measuring phosphorylation of the ligand, or binding of SH2 domains to the ligand, or by assaying for a biological affect on the cell, such as inhibition or stimulation of proliferation, differentiation or migration.

In an embodiment of the method, the substance is an Eph subfamily receptor tyrosine kinase protein, which is not the native receptor tyrosine kinase protein for the ligand, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein. In a further embodiment the part of the protein comprises an extracellular, transmembrane and juxtamembrane domain. In a still further embodiment the part of the protein comprises an extracellular domain.

Another aspect of the invention provides a method for assaying a medium for an agonist or antagonist of a ligand regulatory pathway in a cell which comprises providing a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the surface of the cell, reacting the cell with an Eph subfamily receptor tyrosine kinase protein or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein, and a suspected agonist or antagonist, under conditions which permit the formation of ligand-receptor tyrosine kinase protein complexes on the cell surface, and assaying for ligand-receptor tyrosine kinase protein complexes, for free receptor tyrosine kinase protein, for non-complexed proteins, for activation of the receptor tyrosine kinase protein, or for activation of the ligand.

In an embodiment, activation of the ligand is assayed by measuring phosphorylation of the ligand or binding of SH2 domains to the ligand or by assaying for a biological affect on the cell, such as inhibition or stimulation of proliferation, differentiation or migration.

The invention still further provides a method for affecting neuronal development or regeneration in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein. In an embodiment, the prot in or part of the protein is lacking in a catalytic kinase domain. In another

embodiment, the part of the protein comprises an extracellular, juxtamembrane or transmembrane domain. In a further embodiment, the part of the protein comprises at least one of an extracellular, juxtamembrane and transmembrane domain, preferably an extracellular domain.

In yet another aspect, the invention provides a method for stimulating or inhibiting axonogenesis in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein. In an embodiment, the part of the protein comprises an extracellular domain of an Eph subfamily receptor tyrosine kinase. In a further embodiment, the protein or part of the protein is lacking in a catalytic kinase domain.

The invention also relates to a pharmaceutical composition which comprises a purified and isolated Eph subfamily receptor tyrosine kinase protein or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein for affecting neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient. The part of the protein may comprise an extracellular domain of an Eph subfamily receptor tyrosine kinase, and the protein or part of the protein may be lacking in a catalytic kinase domain.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the amino acid sequences of members of the Eph subfamily of receptor tyrosine kinases, dots indicate spaces introduced in order to optimize alignment, conserved cysteine residues are marked with asterisks and, arrows indicate the boundaries of the catalytic kinase domain;

Figure 2 shows the nucleotide sequence encoding the Nuk tyrosine kinase protein as shown in SEQ ID NO: 1;

Figure 3 shows the amino acid sequence of Nuk tyrosine kinase protein as shown in SEQ ID NO:2 and a schematic diagram of the regions of the Nuk receptor tyrosine kinase protein;

Figure 4 shows a recombinant DNA molecule having a *Nuk*^{1} null mutation obtained by deletion of exon 2, corresponding to codons 29 to 50 as shown in SEQ ID NO: 1;

Figure 5 shows a recombinant DNA molecule encoding the *Nuk*^{2} mutation in the ATP binding region of the kinase domain of Nuk protein, and a lac Z reporter gene;

Figure 6A is a photomicrograph showing a transverse section taken through the brain of heterozygous *Nuk*^{1}/+ mice across the anterior of the frontal lobes;

Figure 6B is a photomicrograph showing a transverse section taken through the brain of homozygous *Nuk*^{1}/*Nuk*^{1} mice across the anterior of the frontal lobes;

Figure 6C is a phot micrograph showing a transverse section taken through the brain of homozygous *Nuk*^{1} /*Nuk*^{1} mice across the anterior of the frontal lobes;

Figure 6D is a photomicrograph showing a transverse section taken through the brain of homozygous *Nuk*^{2} /*Nuk*^{2} mice across the anterior of the frontal lobes (ac=anterior commissure, mt=medial tract);

Figure 7 A is a photomicrograph of a horizontal section taken through the brain of a *Nuk*^{1}/+ mouse across the anterior of the frontal lobes, showing the medial tract of the anterior commissure;

Figure 7B is a photomicrograph of a horizontal section taken through the brain of a homozygous *Nuk*^{1}/*Nuk*^{1} mouse across the anterior of the frontal lobes, showing the absence of the medial tract of the anterior commissure;

Figure 8 shows horizontal sections taken through the brains of *Nuk*^{1}/*Nuk*^{1} (bottom) and *Nuk*^{1}/+ (top) mice injected in one frontal lobe with a fluorescent dye, fast blue;

Figure 9 is a diagram illustrating the fast blue tracing of the temporal lobe;

Figure 10 is a diagram illustrating the axon pathways affected in Nuk/Sek4 double homozygotes;

Figure 11 shows an alignment of the amino acid sequences of ligands of the Eph subfamily of receptor tyrosine kinase proteins, amino acids identical in at least five out of nine proteins are shown in inverse type, the cysteine residues common to all nine proteins are marked by asterisks;

Figure 12 is a diagram showing membrane anchored ligands for Eph subfamily receptor tyrosine kinase proteins; and

Figure 13 is a diagram showing a potential signalling role for Lerks.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present inventors have identified and characterized a novel ligand regulatory pathway that plays a crucial role in cell-cell interactions and axonogenesis in the development and regeneration of the nervous system. The present inventors have determined that Eph subfamily receptor tyrosine kinases activate a ligand regulatory pathway in cells expressing ligands for the Eph subfamily receptor tyrosine kinases.

Expression of an Eph subfamily receptor tyrosine kinase, Nuk, was found to be essential for formation of at least one commissure in the brain, the medial tract of the anterior commissure. In null mice, lacking in Nuk expression the medial tract was found not to form. In *Nuk*^{2}/*Nuk*^{2} mice, expressing a fusion protein comprising the Nuk protein extracellular domain and ß-galactosidase, the medial tract of the anterior commissure formed and was of a normal appearance. Therefore, the extracellular domain of Nuk protein is required for formation of the medial tract of the anterior commissure. Nuk protein did not appear to be expressed in the medial tract of the anterior commissure, but expression was

detected ventrally underlying the commissure. Ligands f Nuk protein are th ught to be expressed in the medial tract of the commissure. Nuk protein also appears to play an important role in the formation of the habenular interpeduncle tract in the brain. Complete formation of the habenular interpeduncle tract was shown to require expression of at least two members of the Eph subfamily of receptor tyrosine kinase proteins and appeared to require expression of Nuk protein having a catalytic kinase domain. Both *Nuk¹/Nuk¹* and *Nuk²/Nuk²* homozygotes exhibit a mild phenotype in the habenular interpeduncle tract, however, this phenotype is more severe in either *Nuk¹/Nuk¹:Sek4/Sek4* and *Nuk²/Nuk2:Sek4/Sek4* double homozygotes.

The invention relates to a method of activating a ligand regulatory pathway in a cell, comprising reacting an Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein with a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the surface of the cell thereby activating the ligand regulatory pathway in the cell.

The term "ligand regulatory pathway" used herein refers to the interactions of an Eph subfamily receptor tyrosine kinase protein with a cell surface ligand for an Eph subfamily receptor tyrosine kinase protein, to form a ligand receptor tyrosine kinase protein complex thereby activating a series of downstream regulatory pathways in the ligand expressing cell that affect the cell, for example by controlling gene expression, cell division, cytoskeletal architecture, cell metabolism, migration, cell-cell interactions and spatial positioning. Examples of such downstream regulatory pathways are the GAP/Ras pathway, the pathway that regulates the breakdown of the polyphosphoinositides through phospholipase C (PLC) and the Src/tyrosine kinase and Ras pathways.

"Eph subfamily receptor tyrosine kinase proteins" refers to proteins of the Eph subfamily which are characterised as encoding a structurally related cysteine rich extracellular domain containing a single immunoglobulin (Ig)-like loop near the N-terminus and two fibronectin III (FN III) repeats adjacent to the plasma membrane. The structure of the extracellular region is thought to determine ligand binding specificity. The intracellular regions contain the juxtamembrane and the catalytic kinase domain. Receptor mediated signal transduction is initiated in the receptor expressing cell by ligand binding to the extracellular domain, which facilitates dimerization of the receptor and autophosphorylation.

Over a dozen members of the Eph subfamily have been identified (van der Geer et al., 1994, *Annu. Rev. Cell. Biol.*. 10:251-237). Examples of Eph family members include mouse Nuk and its homologs Hek5, Cek5 in chickens (Pasquale, *Cell Regulation* 2:523-534, 1991), Sek3 in mice, and Erk in humans; Eek (Chan and Watt, *Oncogene* 6:1057-1061 1991); rat Elk and its homologs including Cek6a in chickens and xEK (Lhotak et al., 1991, Mol. Cell. Biol. 11:2496-2502); human Hek2 and its homol gs including Sek4 in mice and Cek10 in chickens;

and human Htk and its homol gs including Mykl in mice. The Eph family member, *Sek* has been shown to be segmentally expressed in specific rhombomeres of the mouse hindbrain (Nieto *et al., Development* 116:1137-1150, 1992). Other members of the family include Eck (Lindberg and Hunter, 1990, *Mol. Cell Biol.* 10:6316-6324); Ceks 4, 6, 7, 8, 9 and 10 (Pasquale, 1991, *Cell Regulation*, 2:523-534) and Saajadi and Pasquale, 1993, *Oncogene*, 8, 1807-1813); Ehk 1 and 2 (Maisonpierre et al., 1993,*Oncogene*, 8:3277-3288); Myk 1 and 2 (Andres et al., 1994); and Heks 4, 5, 7 (GenBank Accession No. L36644), 8 (GenBank Accession No. L36645) and 11 (GenBank Accession No. L36642) (Fox, et al., 1995, *Oncogene*, 10, 897-905). The amino acid sequences of some known members of the Eph subfamily of receptor tyrosine kinases are described in Fox et al., 1995 (*Oncogne* 10, 897-905) and shown in Figure 1, which is excerpted from Fox et al., 1995, supra. Amino acid sequences for other Eph subfamily receptors can be found in GenBank (e.g. Accession Nos. L25890 (Nuk), X13411 (rat Elk), U07695 (human Htk) and the publications referred to therein).

Preferably, Eph subfamily receptor tyrosine kinases, or parts thereof, which bind to transmembrane ligands are used in the present invention. For example, preferred Eph subfamily receptor tyrosine kinases, or parts thereof, used in the present invention include mouse Nuk and its homologs Hek5, Cek5 in chickens, and Erk; rat Elk and its homologs including Cek6a in chickens and xEK; human Hek2 and its homologs including Sek4 in mice and Cek10 in chickens; and human Htk and its homologs including Mykl in mice.

All the hallmarks of a receptor tyrosine kinase of the Eph subfamily family are exemplified in Nuk protein, including 20 cysteine residues whose position is conserved in the extracellular domain of Eph family members (bold type, Figure 3), an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III; between Nuk amino acids residues 330-420 and 444-534). The Ig-like domain of Nuk contains specific residues (Cys^{70}, Trp^{80}, Cys^{115}) known to be conserved in the Ig superfamily (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988).

The cartoon in Figure 3 shows the location of the various domains of Nuk protein. Following a 26 amino acid hydrophobic signal peptide, the Nuk protein extracelluar domain is composed of an Ig-like domain and two FN III repeats. The Nuk protein extracelluar domain also contains 20 cysteines whose position is conserved in the Eph family (Lhotak *et al., Mol. Cell. Biol.* 11:2496-2502, 1991). A hydrophobic transmembrane domain divides the Nuk protein into approximately two halves, a 548 amino acid extracelluar region and a 419 amino acid cytoplasmic region which contains a tyrosine kinase catalytic domain.

Nuk is most highly related to the full length amino acid sequence of human Hek5 and also to chicken Cek5 (96% identity; Pasquale, *Cell Regulation* 2:523-534, 1991) and to short PCR products of mRNA from rats (*Tyro* 5; Lai and Lemke, *Neuron* 6:691-704, 1991) and humans (*Erk*; Chan and Watt, *Oncogene* 6:1057-1061 1991). The close identity between *Nuk* and *Cek5* suggest they represent the mammalian and avian orthologs of the same progenitor

gene. The absence of full length cDNAs f r *Tyro* 5 and *Erk* precludes the determination of whether these sequences correspond to the same or a closely related but different gene.

It will be appreciated that the Eph subfamily receptor tyrosine kinase protein for use in activating a ligand regulatory pathway, as described herein, may be an isoform or a part of the protein having at least 20 contiguous amino acids of the protein. An isoform contains the same number and kinds of amino acids as the protein, but the isoform has a different molecular structure. The isoforms contemplated for use in the methods of the invention are isoforms having the same functional properties as the Eph subfamily receptor tyrosine kinase proteins.

In a preferred embodiment, the part of the protein having at least 20 contiguous amino acids comprises an Eph subfamily tyrosine kinase protein, preferably Nuk, lacking a catalytic kinase domain. For example, the part of the protein containing at least one of the extracellular domain, the transmembrane domain and the juxtamembrane domain or parts thereof, preferably, the extracellular domain is used in the methods herein.

The extracellular domain is characterised by a cysteine rich region, whose position is conserved in the extracellular domain of Eph family members an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III). Extracellular domains of Eph subfamily receptor tyrosine kinase proteins may be identified based on the above-noted features and based on a comparison of the amino acid sequences of the extracellular domains of known Eph subfamily receptor tyrosine kinase proteins. The extracellular domain may be generally defined as the region extracellular to the transmembrane domain, which is indicated in bold underline in Figure 1.

The protein may also be a protein having substantial sequence identity with the sequence of an Eph subfamily receptor tyrosine kinase protein. The term "sequence having substantial identity" means those amino acid sequences having slight or inconsequential sequence variations from the sequence of an Eph subfamily receptor tyrosine kinase protein. The variations may be attributable to local mutations or structural modifications. Suitable proteins may have over 95%, preferably over 97%, most preferably over 99% identity with an Eph subfamily receptor tyrosine kinase protein.

An Eph subfamily receptor tyrosine kinase or part thereof, may be selected for use in the present invention based on the nature of the ligand which is targeted or selected. The selection of a particular ligand and complementary Eph subfamily receptor tyrosine kinase in the method of the invention will allow for the identification of specific substances that affect a ligand regulatory pathway.

An Eph subfamily receptor tyrosine kinase or part thereof may be prepared from Eph subfamily receptor tyrosine kinase proteins isolated from cells which are known to express the proteins. Alternatively the protein or part of the protein may be prepared using recombinant DNA methods known in the art. By way of example, nucleic acid molecules

having a sequence which codes f r an Eph subfamily receptor tyrosine kinase protein, or a part of the protein may be prepared and incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein or part thereof. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses, so long as the vector is compatible with the host cell used.

Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate transcription and translation elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcriptional and translation elements may be supplied by the native receptor tyrosine kinase protein and/or its flanking regions.

The recombinant molecules may also contain a reporter gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule. Examples of reporter genes are genes encoding a protein such as ß-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. In a preferred embodiment, the reporter gene is *lac Z*. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as ß-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase.

Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation etc. Methods for transforming transfecting, etc. host cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference and see the detailed discussion below).

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells.

The Eph subfamily receptor tyrosine kinase protein or parts thereof may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in

homogenous solution (H ubenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

Conjugates of the protein, or parts thereof, with other molecules, such as proteins or polypeptides, may be prepared and used in the methods described herein. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. Thus, fusion proteins may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of an *Eph* subfamily receptor tyrosine kinase protein or parts thereof, and the sequence of a selected protein or marker protein with a desired biological function. The resultant fusion proteins contain Eph subfamily receptor tyrosine kinase protein or a part thereof fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins and parts thereof such as the constant region of immunoglobulin γ1, and lymphokines such as gamma interferon, tumor necrosis factor, IL-1, IL-2,IL-3, Il-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1 and G-CSF.

Sequences which encode the above-described proteins may generally be obtained from a variety of sources, including for example, depositories which contain plasmids encoding sequences including the American Type Culture Collection (ATCC, Rockville Maryland), and the British Biotechnology Limited (Cowley, Oxford England). Examples of such plasmids include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon,) ATCC Nos. 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contains a sequence which encodes Interleukin-1ß), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC Nos. 57592 (which contains sequences encoding Interleukin-4). ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6.

The Eph subfamily receptor tyrosine kinase protein, isoforms or parts thereof, used in the method of the invention may be insolubilized. For example, the receptor protein or part thereof, preferably the extracellular domain, may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, liposomes, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized receptor tyrosine kinase protein may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The receptor tyrosine kinase protein or parts thereof may also be expressed on the surface of a cell using the methods described herein.

Ligands for Eph subfamily receptor tyrosine kinases may be identified based on homology with known ligands and based on their interaction with the extracellular domain of Eph subfamily receptor tyrosine kinases. At least seven ligands for Eph subfamily receptor tyrosine kinases have been identified, all of which are membrane anchored via either a GPI linkage or transmembrane domain (see Figure 12), including B61 (Holzmann et al., 1990, *Mol. Cell Biol.* 10: 5830-5838 and Bartley et al., 1994 *Nature* 368:558-560), also known as LERK-1 (Beckmann et al., 1994, *EMBO J.* 13:3757-3762 and Davis et al., 1994 *Science* 266, 816-819), LERK-2 (Beckmann et al., 1994, supra, and Davis et al., 1994 supra, also known as Eplg-2 (Fletcher et al, 1994, *Oncogne* 9:3241-3247), Cek5 ligand, the chicken homolog of Lerk-2 and Elk-L, (Shao et al., 1994, *J. Biol. Chem.* 269:26606-26609), ELF-1 (Cheng and Flanagan, 1994, *Cell*, 79:157-168), EHK1-L (Davis et al., 1994, supra), also known as LERK-3 (Kozlosky et al., 1995 *Oncogne* 10:299-306) and LERK-4 (Kozlosky et al., 1994, supra) ELF-1, AL-1/RAGS (GPI-anchored, Drescher, et al., 1995, Cell, 82:359-370), LERK-4, HTKL/ELF-2/Lerk5, LERK-2/CEK5-L/ELK-L (Tessier-Lavigne, M., 1995, supra). Ligands of Eph subfamily receptor tyrosine kinases show significant homology with each other. An alignment of the amino acid sequences of ligands of Eph subfamily receptor tyrosine kinases are shown in Figure 11 (excerpted from Drescher, et al., 1995, supra. Ligands for the Eph subfamily receptor tyrosine kinases are known to show promiscuous interactions with different Eph subfamily receptors (Brambilla et al., 1995, EMBO J. 14:3116-3126).

In an embodiment of the invention, the ligands are ligands which are membrane anchored via a transmembrane domain. Preferably, the selected ligands are Elk-L/LERK2/Efl-3/Cek5-L; hHtk-L/ELF-2/LERK5 (Tessier-Lavigne, M., 1995, Cell 82:345-348), and hElk-L3/Elf-6. These ligands have highly conserved cytoplasmic reions with multiple potential sites for phosphorylation. The amino acid sequences for hElk-L3, hHtk-L and hElk-L, and the extracellular domains of the ligands can be found in GenBank (e.g. Accession Nos. L38734 (Htk) and L37361 (Efl-3)).

In the methods of the invention to activate a ligand regulatory pathway in a cell, the ligand should be expressed on the surface of the cell. Preferably, the cell is one which expresses native ligand. However, it will be appreciated that the invention also contemplates chimeric cells expressing a recombinant ligand.

The invention also provides a method for identifying a substance which is capable of binding to a ligand for an Eph subfamily receptor tyrosine kinase and activating a ligand regulatory pathway in a cell, comprising reacting a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase with at least one substance which potentially can bind with the ligand, under conditions which permit the formation of substance-ligand complexes,

and assaying for substance-ligand complexes, for free substance, for non-complexed ligands, or for activation of the ligand.

Activation of the ligand may be assayed by measuring phosphorylation of the ligand, binding of SH2 domains to the ligand, and where the ligand is expressed on a cell surface, by assaying for a biological affect on the cell, such as inhibition or stimulation of proliferation, differentiation or migration. SH2-domains of cytoplasmic signalling proteins are known to bind to phosphorylated receptor tyrosine kinase proteins. In particular, the SH2 domains of $p21^{ras}$ GTPase-activating protein (GAP), Src, and phosphoinositide-specific phospholipase C (PLCγ) may bind an Eph subfamily receptor tyrosine kinase protein. SH2 domains of cytoplasmic signalling proteins may bind to phosphorylated ligands to mediate the interactions of the phophorylated ligand with signalling proteins of the downstream regulatory pathways in the cell.

Upon binding of a ligand having an intracellular domain (e.g. Lerks such as Lerk2 and Lerk5) to an Eph subfamily receptor, a signal transduction event in the ligand expressing cell may be initiated. This could occur by activation of one or more cytoplasmic tyrosine kinases which would phosphorylate the intracellular domain of the ligand, which would then lead to the binding of SH2 domain-containing proteins to the phosphorylated activated ligand. A diagram of a potential signalling role for Lerks is shown in Figure 13.

In an embodiment, of the method, the substance is an Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein. In a further embodiment the part of the protein comprises an extracellular domain. In a preferred embodiment, the substances is an Eph subfamily receptor tyrosine kinase which is not the native receptor tyrosine kinase for the ligand.

Conditions which permit the formation of substance-ligand complexes may be selected having regard to factors such as the nature and amounts of the substance and the ligand.

The substance-ligand complex, free substance or non-complexed ligand may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation,absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the ligand or the substance, or a labelled ligand, or a labelled substance may be utilized. Antibodies, receptor protein or substance may be labelled with a detectable substance as described above.

The substance used in the method of the invention may be insolubilized. For example, the receptor protein or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid c polymer, amino acid copolymer,

ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The substance may also be expressed on the surface of a cell using the methods described herein. Where the substance is expressed on the surface of a cell the presence of a substance which can bind to and be activated by the receptor tyrosine kinase protein may be identified by assaying for activation of the substance or by assaying for a biological affect on the cell.

The above mentioned methods of the invention may be used to identify substances which bind with ligands of the Eph subfamily of receptor tyrosine kinase proteins, thereby activating a ligand regulatory pathway in a cell, particularly those involved in neuronal development, axonal migration, pathfinding and regeneration. Identification and isolation of such substances will permit studies of the role of the substance in the developmental regulation of axonogenesis and neural regeneration, and permit the development of substances which affect these roles, such as functional or non-functional analogues of the extracellular domain of an Eph subfamily receptor tyrosine kinase. It will be appreciated that such substances will be useful as pharmaceuticals to modulate axonogenesis, nerve cell interactions and regeneration to treat conditions such as neurodegenerative diseases and cases of nerve injury.

Substances which bind to and activate the ligand may be identified by assaying for protein tyrosine kinase activity i.e. by assaying for phosphorylation of the tyrosine residues of the ligand, using known techniques such as those using anti-phosphotyrosine antibodies and labelled phosphorous. For example, immunoblots of the complexes may be analyzed by autoradiography (^{32}P-labelled samples) or may be blocked and probed with antiphosphotyrosine antibodies as described in Koch, C.A. et al., 1989 (Mol. Cell. Biol. 9, 4131-4140).

Substances which bind to and activate the ligand may also be assayed by assaying for a biological affect on the cell, for example inhibition or stimulation of cell proliferation, differentiation and migration. Substances which bind to and activate the ligand will include Eph subfamily receptor tyrosine kinase proteins and portions of the proteins. The method will permit identification of the minimum amino acid sequence of the protein which is required for ligand binding and activation.

The invention further relates to a method for assaying a medium for an agonist or antagonist of a ligand regulatory pathway in a cell which comprises providing a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the cell surface, reacting the cell with an Eph subfamily receptor tyrosine kinase protein or part of a protein and a suspected agonist or antagonist under conditions which permit the formation of ligand-

receptor tyrosine kinase protein complexes on the cell surface, and assaying for ligand-receptor tyrosine kinase protein complexes, for free receptor tyrosine kinase protein, for non-complexed proteins, for activation of the receptor tyrosine kinase protein, or for activation of the ligand.

Substances which activate the ligand regulatory pathway, such as Eph subfamily receptor tyrosine kinase proteins or parts thereof, and agonists or antagonists of the ligand regulatory pathway may be used for affecting neuronal development or regeneration in a mammal. The substances, agonists and antagonists may be used to stimulate or inhibit neuronal development, regeneration and axonal migration associated with neurodegenerative conditions and conditions involving trauma and injury to the nervous system, for example Alzheimer's disease, Parkinson's disease, Huntington's disease, demylinating diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, bacterial and viral infections of the nervous system, deficiency diseases, such as Wernicke's disease and nutritional polyneuropathy, progressive supranuclear palsy, Shy Drager's syndrome, multistem degeneration and olivo ponto cerebellar atrophy, peripheral nerve damage, trauma and ischemia resulting from stroke.

The ability of substances, agonists, and antagonists identified using the methods of the invention to affect neuronal development or regeneration and to stimulate nerve regeneration, may be confirmed in an animal model having an injured peripheral nervous system. Examples of mammals having an injured peripheral nervous system include animals having damaged axons, such as axotomized facial neurons (Sendtner et al. Nature, 345, 440-441, 1990), neurodegenerative conditions (for example, the MPTP model as described in Langston J.W. et al., Symposium of Current Concepts and Controversies in Parkinson's Disease, Montebello, Quebec, Canada, 1983 and Tatton W.G. et al., Can. J. Neurol. Sci. 1992, 19), and traumatic and non-traumatic peripheral nerve damage (for example, animal stroke models such as the one described in MacMillan et al. Brain Research 151:353-368 (1978)).

The present invention thus provides a method for affecting neuronal development or regeneration in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein, or a substance identified using the methods of the invention. The invention also contemplates a method for stimulating or inhibiting axonogenesis in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein, or a substance identified using the methods of the invention.

The invention still further relates to a pharmaceutical composition which comprises a purified and isolated Eph subfamily receptor tyrosine kinase protein or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein, or a

substance identified using the methods of the invention, for affecting neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical compositions may be used to stimulate or inhibit neuronal development, regeneration and axonal migration associated with neurodegenerative conditions and conditions involving trauma and injury to the nervous system as described above.

The compositions of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of an Eph subfamily receptor tyrosine kinase protein may vary according to factors such as the condition, age, sex, and weight of the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (e.g., protein) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration inhalation, transdermal application or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. The pharmaceutical compositions of the invention can be for oral, local, inhalant or intracerebral administration. Preferably, the pharmaceutical compositions of the invention are administered directly to the peripheral or central nervous system, for example by administration intracerebrally.

The pharmaceutical composition of the invention can be administered to a subject in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as microporous or solid beads or liposomes. The term "pharmaceutically acceptable carrier" as used herein is intended to include diluents such as saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) J. Neuroimmunol 7:27). The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The pharmaceutically acceptable carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., antibody) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the composition may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is contemplated that the pharmaceutical compositions may be administered locally to stimulate axonogenesis and pathfinding in areas of the body in need thereof, for example in areas of local nerve injury or in areas where normal nerve pathway development

has not occurred. It is also contemplated that the pharmaceutical c mpositions may be placed in a specific orientation or alignment along a presumptive pathway to stimulate axon pathfinding along that line, for example the pharmaceutical compositions may be present on microcarriers laid down along the pathway. In an embodiment, the pharmaceutical compositions may be used to stimulate formation of connections between areas of the brain, such as the area between the two hemispheres or between the thalamus and ventral midbrain. In an embodiment, the compositions may be used to stimulate formation of the medial tract of the anterior commissure or the habenular interpeduncle.

It is also contemplated that the pharmaceutical compositions of the invention may comprise cells or viruses, preferably retroviral vectors, transformed with nucleic acid molecules encoding a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein, or a substance identified using the methods of the invention, such that they express the protein, isoform, or a part of the protein, preferably the extracellular domain, or substance *in vivo*. Viral vectors suitable for use in the present invention are well known in the art including recombinant vaccinia viral vectors (U.S. Patent Nos. 4,603,112 and 4,769,330), recombinant pox virus vectors (PCT Publication No. WO 89/01973), and preferably, retroviral vectors ("Recombinant Retroviruses with Amphotropic and Ecotropic Host Ranges," PCT Publication No. WO 90/02806; "Retroviral Packaging Cell Lines and Processes of Using Same," PCT Publication No. WO 89/07150; and "Antisense RNA for Treatment of Retroviral Disease States," PCT Publication No. WO 87/03451). The compositions containing cells or viruses may be directly introduced into a subject as described herein. Nucleic acid molecules encoding a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein, or a substance identified using the methods of the invention, may also be introduced into a subject using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of nucleic acids into liposomes. They may also be delivered in the form of an aerosol or by lavage.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

The following materials and methods were utilized in the investigations outlined in the examples:

Cloning of *Nuk* The coding region of *Nuk* was cloned using the partial λQ1, *Nuk* cDNA insert to probe a λgt10 expression library constructed from a mouse erythroleukemia cell line by screening with anti-phosphotyrosine antibodies (Ben-David et al., EMBO 10:317-325, 1991). Cloning of *Nuk* was carried out as described in Henkemeyer et al., 1994 (*Oncogene* 9:1001-1014) and in co-pending International Application PCT CA95/00254 and co-pending application serial No. 08/235,407, which are incorporated herein by reference. The

nucleotide sequence encoding Nuk is shown in Figure 2 (SEQ. ID. NO: 1) and the amino acid sequence of Nuk protein is shown in Figure 3 (SEQ. ID. NO: 2).

Generation of Loss of Function *Nuk* mutant

A loss of function mutation in *Nuk*, designated *Nuk*^{1} was generated in embryonic stem cells, and germline transmission of the null allele was obtained as described in co-pending International Application PCT CA95/00254 and co-pending application serial No. 08/235,407.

Briefly, the null mutation was obtained by deletion of exon 2, corresponding to codons 29 to 50, as shown in Figure 4. To obtain germ line transmission of the mutation *Nuk*+/- embryonic stem cell lines (ES) were aggregated with 8 cell embryos *in vitro* and the resulting blastocysts were transferred into recipient females. Upon birth, animals chimeric for ES and embryonic stem cells were recovered by scoring for eye pigment and coat colour. Breeding of these "aggregation chimeras" confirmed that the germ line of at least one founder mouse is derived completely from the ES cells. Adult mice homozygous for the mutation did not express Nuk protein.

Generation of a *Nuk-lac Z* fusion chimeric receptor mutant

A targeted mutation, designated *Nuk*^{2} was generated in the *Nuk* gene as described in co-pending International Application PCT CA95/00254 and co-pending application serial No. 08/235,407 and shown in Figure 5. A *pPNT-LOX-Nuk*^{2} gene trap vector was used to delete the GXGXXG ATP binding region of the kinase domain (amino acids 623-707,) to create a Nuk-lac Z fusion receptor in ES cells. Chimeric animals were prepared as described above, by aggregating the ES cells with 8 cell CD1 embryos.

Animals generated with the *Nuk*^{2} mutation provided *Nuk* expressing cells staining for ß-galactosidase activity, providing a convenient marker for Nuk-positive cells in both heterozygous and homozygous backgrounds. The *Nuk*^{2} mutation led to the expression of a Nuk-beta galactosidase fusion protein in mouse heterozygous embryos, detected by a blue/green colour.

EXAMPLE 1

The role of Nuk protein, the extracellular domain of Nuk protein and the catalytic kinase domain of Nuk protein were investigated as follows. Loss of function *Nuk* mutant mice, designated *Nuk*^{1} were prepared as described herein. These mice may also be referred to as null mice as they do not express Nuk protein. *Nuk-lac Z* fusion chimeric receptor mutant mice, designated *Nuk*^{2} were prepared as described above. These mice express a fusion protein having the entire extracellular domain of Nuk, but lacking in the Nuk catalytic kinase domain, which is replaced by ß-galactosidase. All mice, exhibited apparently normal appearance and behaviour.

To analyze the brains of Nuk mutant mice, specimens were dissected and fixed in 4% paraformaldehyde in PBS. The fixed specimens were either embedded in paraffin and

sectioned on a microtome r cryprotected in 30% sucrose and sectioned using a cryostat to obtain serial sections.

Serial sections were taken of a number of brains of heterozygous control and both *Nuk*^{1} and *Nuk*^{2} homozygous embryos at E14.5 to E18.5 days of embryonic development and of newborn and adult mice at 1 to 1.5 years of age. 6 to 30 μm thick coronal or horizontal sections were prepared and viewed on a compound microscope under bright field or polarized light. Figures 6A, 6B, 6C and 6D show photomicrographs of horizontal sections taken across the anterior of the temporal lobes at the level of the anterior commissure and pars posterior medial tract, which connects the frontal lobes. In heterozygous *Nuk*^{1}/+ mice the pars posterior medial tract, and the pars anterior tract of the anterior commissure are clearly visible (Figure 6A) and appear the same as in wild type mice. Serial sections show that the pars posterior medial tract forms a continuous tract between the two frontal lobes. The entire medial tract is not visible in Figure 6A due to the plane of the section.

The presence of a continuous pars posterior medial tract communicating between the frontal lobes, was confirmed by dye injection experiments, which are illustrated diagramatically in Figure 9. Briefly, a fluorescent dye (fast blue) was injected into one temporal lobe of anaesthetized adult mice, either heterozygous or homozygous for the *Nuk*^{1} mutation, approximately one year old, through standard surgical techniques. Mice were revived and the fast blue was allowed to travel through the axons of the temporal neurons that received dye for 2 days, after which the mice were sacrificied, perfused with fixative, and the brains were collected and post-fixed. After cryoprotection in 30% sucrose, serial sections were prepared and the brain sections were viewed by fluoresence microscopy. Where the dye was found to have been transported across to the opposite frontal lobe, the presence of an intact medial tract was confirmed.

In homozygous *Nuk*^{1}/*Nuk*^{1} null mice the pars posterior medial tract was found to be absent as shown in Figures 6B, 6C and 7B. Absence of the medial tract was confirmed by the inability of dye injected into one frontal lobe to cross to the opposite frontal lobe as shown in Figure 8 (bottom). Absolutely no label was detected in the opposite frontal lobe, even when large amounts of dye were injected to maximize labelling. In *Nuk*^{1}/+ mice, however, small amounts of dye were sufficient to produce visible labelling in the opposite frontal lobe, as shown in Figure 8 (top). Labeling was detected in the medial tract of *Nuk*^{1}/+ mice but not in *Nuk*^{1}/*Nuk*^{1} mice. This directly shows that expression of Nuk protein is required for the formation of the medial tract.

In homozygous *Nuk*^{2}/*Nuk*^{2} mice the medial tract was found to be present, as shown in Figure 6D and was shown by dye injection to form a continuous connection between the frontal lobes, as in the wild type and *Nuk*^{1}/+ heterozygotes. This surprisingly indicates that the extracellular domain of Nuk, in the absence of the catalytic kinase domain, is sufficient for formation of the medial tract. This is believed to be the first showing of a functional role

for the extracellular domain of a receptor tyrosine kinase which is independent of the catalytic kinase domain. A role for the transmembrane and juxtamembrane domains of Nuk protein cannot be ruled out as the chimeric Nuk-ß-galactosidase fusion protein has these domains in addition to the extracellular domain.

In view of the importance of Nuk protein in the formation of the pars posterior medial tract, a detailed study of the expression of *Nuk* in this region of the brain was made by examining serial sections from the brains of Nuk^2/Nuk^2 homozygous mice, which express a fusion protein comprising the Nuk extracellular transmembrane and juxtamembrane domains and ß-galactosidase, which can readily be detected in sections based on a blue green coloration, as described herein. Sections were taken from the brains of Nuk^2/Nuk^2 mice and newborn pups and from embryos at various stages of gestation.

Nuk was not found to be expressed in the pars posterior medial tract of embryonic or adult Nuk^2/Nuk^2 mice. *Nuk* expression was absent dorsal to the medial tract but apparent in the cells ventral to and underlying the medial tract.

Nuk was generally found to be widely expressed in the brain, with an apparent increase in level posteriorly. Peripheral axons were found to express high levels of *Nuk*. In particular, the retinal ganglia cells of the eyes exhibited intense blue/green staining. The olfactory receptor neurons, the trigeminal ganglia and associated sensory whisker roots were also found to express *Nuk*. The corpus callosum, the thick stratum of transversely-directed nerve fibres which connects the two hemispheres of the brain, was also stained for *Nuk* expression.

Further information about the role of Nuk protein in axonal pathfinding was obtained from examining the brains of mice having double mutations in *Nuk* and in *Sek4*, another member of the *Eph* subfamily of receptor tyrosine kinases. Mice bearing a Sek4 null mutation were prepared (Klein and Orioloi, European Molecular Biology Laboratory, Heidelberg, Germany). The *Sek4* null mice, similar to the *Nuk* null mice, exhibited no obvious morphological or behavioral defects. However, $Nuk^{1}/Sek4$ double homozygous mutants died at birth. Nuk^2/Sek^4 mice survived up to 3 months, confirming that Nuk protein plays a crucial role which is independent of its catalytic kinase domain.

An examination of coronal sections of the brains of newborn Nuk^1/Sek^4 mice showed that, in addition to the anterior commissure defect found in $Nuk^1/-$ mice, the corpus callosum and habenular interpeduncle tracts were severly affected and failed to develop properly. The axon pathways affected in the *Nuk/Sek* double homozygotes is illustrated in Figure 10. The fibres of the anterior commissure appeared to be misdirected and oriented to the ventral-most floor of the brain. In addition, the fibres of the corpus callosum had not joined up across the midline, but had piled up against the lateral ventricles. *Nuk,-Lac Z* expression, based on blue/green staining, was detected in the mid line of the corpus callosum. The habenular interpeduncle tract which connects the thalamus to the ventral midbrain, was

defective in *Nuk²/Sek4* and *Nuk¹/Sek4*. Careful analysis of Nuk protein using anti-Nuk antibodies and *lac Z* staining of *Nuk²/Nuk²* embryos showed that, during development, *Nuk* expression appears in the ventral midbrain and progresses towards the thalamus and axon migration occurred in the opposite direction, i.e. from the thalamus toward the ventral mid brain. This axon migration was dependent on the expression of Nuk protein having a catalytic kinase domain.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated by those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The following sequence listings form part of the application.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: **Method of Activating a Novel Ligand Regulatory Pathway**

(iii) NUMBER OF SEQUENCES: 2

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(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kurdydyk, Linda M.
(B) REGISTRATION NUMBER: 34,971
(C) REFERENCE/DOCKET NUMBER: 3153-196

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (416) 364-7311
(B) TELEFAX: (416) 361-1398
(C) TELEX: 06-23115

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3105 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10 cDNA library
(B) CLONE: Combined PnUKRACE A2 and K2 AND cDNA clones

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
(B) MAP POSITION: near the ahd-1 mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTGGGCTGGA TGGTACATCC CCCATCAGGG TGGGAAGAGG TGAGCGGCTA CGACGAGAAC 180

ATGAACACTA TCCGTACCTA CCAGGTGTGC AATGTCTTTG AGTCAAGCCA GAACAACTGG 240

CTGCGGACCA AATTCATCCG GCGCCGTGGC GCCCACCGTA TCCACGTGGA GATGAAGTTC 300

TCGGTGCGTG ACTGCAGCAG CATTCCCAGC GTGCCGGGCT CCTGCAAGGA GACCTTCAAC 360

CTCTACTACT ATGAGGCTGA TTTTGACTTA GCCACCAAAA CCTTTCCCAA CTGGATGGAG 420

AATCCGTGGG TGAAGGTGGA CACCATCGCG GCCGATGAGA GCTTCTCTCA GGTGGACCTG 480

GGTGGCCGCG TCATGAAAAT CAACACTGAG GTGCGAAGCT TCGGTCCTGT GTCCCGCAAT 540

GGTTTCTACC TGGCCTTCCA GGACTACGGC GGCTGTATGT CCCTCATTGC TGTGCGCGTC 600

TTCTACCGGA AGTGCCCCCG AATCATCCAG AATGGTGCCA TCTTCCAGGA GACACTATCG 660

GGGGCTGAGA GCACTTCGCT GGTGGCAGCT CGGGGCAGCT GCATCGCCAA TGCTGAAGAA 720

GTGGACGTGC CCATCAAACT CTACTGTAAC GGGGACGGCG AATGGCTGGT GCCCATCGGT 780

CGCTGCATGT GCAAGGCGGG CTTCGAGGCT GTGGAGAACG GCACCGTCTG CCGAGGTTGT 840

CCATCAGGAA CCTTCAAGGC CAACCAAGGG GACGAAGCCT GCACCCACTG TCCCATCAAC 900

AGCCGCACCA CCTCTGAGGG TGCCACCAAC TGTGTATGCC GCAACGGCTA CTACAGGGCC 960

GACCTGGACC CCTTAGACAT GCCTTGCACA ACCATCCCCT CTGCGCCCCA GGCTGTGATC 1020

TCCAGCGTCA ACGAGACATC CCTCATGCTA GAGTGGACCC CACCCCGAGA CTCCGGGGGT 1080

CGCGAGGATC TTGTTTACAA CATCATCTGC AAGAGCTGTG GCTCCGGCCG GGGCGCATGC 1140

ACGCGCTGCG GGGACAACGT GCAGTACGCG CCCCGCCAGC TGGGCCTGAC TGAGCCGCGC 1200

ATCTACATCA GTGACCTGCT GGCACACACG CAGTACACCT TCGAGATCCA GGCCGTGAAT 1260

GGTGTGACCG ACCAGAGTCC CTTCTCACCT CAGTTCGCCT CTGTGAACAT CACCACCAAC 1320

CAAGCAGCAC CATCGGCCGT GTCCATCATG CACCAGGTGA GCCGCACTGT GGACAGCATC 1380

ACCCTGTCGT GGTCCCAGCC AGACCAGCCC AACGGTGTGA TCCTGGACTA CGAGCTGCAG 1440

TACTATGAGA AGGAGCTCAG TGAGTACAAC GCCACGGCCA TAAAAAGCCC CACCAACACA 1500

GTCACTGTGC AGGGCCTCAA AGCCGGCGCC ATCTATGTCT TCCAGGTGCG GGCACGCACC 1560

GTTGCAGGCT ATGGGCGCTA CAGTGGCAAG ATGTACTTCC AAACCATGAC AGAAGCCGAG 1620

TACCAGACCA GCATCAAGGA AAAGCTACCC CTCATCGTTG GCTCCTCCGC CGCCGGCTTA 1680

GTCTTCCTCA TCGCTGTGGT CGTCATTGCC ATCGTATGTA ACAGACGGGG GTTTGAGCGT 1740

GCCGACTCAG AGTACACGGA CAAGCTACAG CACTACACCA GCGGACACAT GACCCCAGGC 1800

ATGAAGATCT ATATAGATCC TTTCACCTAT GAAGATCCTA ATGAGGCAGT GCGGGAGTTT 1860

GCCAAGGAAA TTGACATCTC CTGTGTCAAG ATTGAGCAGG TGATTGGAGC AGGGGAATTT 1920

GGTGAGGTCT GCAGTGGCCA TTTGAAGCTG CCAGGCAAGA GAGAGATCTT TGTAGCCATC 1980

AAGACCCTCA AGTCAGGATA CACGGAGAAA CAGCGCCGGG ACTTCCTGAG TGAGGCATCC 2040

ATCATGGGCC AGTTCGACCA CCCCAATGTC ATCCATCTGG AAGGGGTTGT CACCAAGAGC 2100

ACACCTGTCA TGATCATCAC TGAATTCATG GAGAATGGAT CTCTGGACTC CTTCCTCCGG 2160

CAAAATGATG GGCAGTTCAC AGTCATCCAA CTGGTGGGCA TGCTGAGGGG CATTGCAGCC 2220

GGCATGAAGT ACCTGGCGGA CATGAACTAC GTGCACCGTG ACCTTGCTGC TCGAAACATC 2280

CTCGTCAACA GTAACCTGGT GTGTAAGGTG TCTGACTTTG GGCTCTCACG CTTCCTGGAG 2340

GATGACACGT CTGACCCCAC CTATACCAGC GCTCTGGGTG GGAAGATCCC CATCCGTTGG 2400

ACGGCACCGG AAGCCATCCA GTACCGGAAA TTCACCTCGG CCAGTGATGT GTGGAGCTAT 2460

GGCATCGTCA TGTGGGAGGT GATGTCCTAC GGGGAACGAC CCTACTGGGA CATGACCAAT 2520

CAAGACGTAA TCAACGCCAT TGAACAGGAC TACAGACTAC CTCCGCCCAT GGACTGCCCT 2580

AGCGCCCTGC ACCAGCTCAT GCTGGACTGC TGGCAGAAGG ACCGCAACCA CCGGCCCAAG 2640

TTCGGCCAGA TTGTCAACAC GCTGGACAAG ATGATCCGAA ACCCCAACAG CCTCAAAGCC 2700

ATGGCACCCC TGTCCTCTGG CATCAACCTG CCACTGCTGG ACCGCACGAT ACCGGACTAC 2760

ACCAGCTTTA ACACAGTGGA TGAGTGGCTA GAGGCCATCA AGATGGGCCA GTACAAGGAG 2820

AGCTTTGCCA ACGCCGGCTT CACCTCTTTC GACGTTGTAT CTCAGATGAT GATGGAGGAC 2880

ATTCTCCGCG TTGGGGTCAC TCTAGCTGGC CACCAGAAAA AAATCCTGAA CAGTATCCAG 2940

GTGATGCGGG CCCAGATGAA CCAGATCCAG TCTGTAGAGG TTTGACATTC GCCTGCCTCG 3000

GTTCTCCTCT TCCTCCACGC CGCCCCTGAG CCCCTACGTC GGTCCCTGCT GCTCTGTCAC 3060

TGCAGGTCAG CACTGCCAGG AGGCCACAGA CAACAGGAAG ACCAA 3105

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 994 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mus musculus
(D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lamda gt10 cDNA library
(B) CLONE: Combined pNukRACE A2 and K2 and cDNA clones

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
(B) MAP POSITION: near the ahd-1 mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ala Arg Val Pro Val Leu Pro Gly Leu Asp Gly Ser Phe Cys
1 5 10 15

Trp Leu Leu Leu Leu Pro Leu Leu Ala Ala Val Glu Glu Thr Leu Met
20 25 30

Asp Ser Thr Thr Ala Thr Ala Glu Leu Gly Trp Met Val His Pro Pro
35 40 45

Ser Gly Trp Glu Glu Val Ser Gly Tyr Asp Glu Asn Met Asn Thr Ile
50 55 60

Arg Thr Tyr Gln Val Cys Asn Val Phe Glu Ser Ser Gln Asn Asn Trp
65 70 75 80

Leu Arg Thr Lys Phe Ile Arg Arg Arg Gly Ala His Arg Ile His Val
85 90 95

Glu Met Lys Phe Ser Val Arg Asp Cys Ser Ser Ile Pro Ser Val Pro
100 105 110

Gly Ser Cys Lys Glu Thr Phe Asn Leu Tyr Tyr Tyr Glu Ala Asp Phe
115 120 125

Asp Leu Ala Thr Lys Thr Phe Pro Asn Trp Met Glu Asn Pro Trp Val
130 135 140

Lys Val Asp Thr Ile Ala Ala Asp Glu Ser Phe Ser Gln Val Asp Leu
145 150 155 160

Gly Gly Arg Val Met Lys Ile Asn Thr Glu Val Arg Ser Phe Gly Pro
165 170 175

Val Ser Arg Asn Gly Phe Tyr Leu Ala Phe Gln Asp Tyr Gly Gly Cys
180 185 190

Met Ser Leu Ile Ala Val Arg Val Phe Tyr Arg Lys Cys Pro Arg Ile
195 200 205

Ile Gln Asn Gly Ala Ile Phe Gln Glu Thr Leu Ser Gly Ala Glu Ser
210 215 220

Thr Ser Leu Val Ala Ala Arg Gly Ser Cys Ile Ala Asn Ala Glu Glu
225 230 235 240

Val Asp Val Pro Ile Lys Leu Tyr Cys Asn Gly Asp Gly Glu Trp Leu
245 250 255

Val Pro Ile Gly Arg Cys Met Cys Lys Ala Gly Phe Glu Ala Val Glu
260 265 270

Asn Gly Thr Val Cys Arg Gly Cys Pro Ser Gly Thr Phe Lys Ala Asn
275 280 285

```
Gln Gly Asp Glu Ala Cys Thr His Cys Pro Ile Asn Ser Arg Thr Thr
     290                 295                 300

Ser Glu Gly Ala Thr Asn Cys Val Cys Arg Asn Gly Tyr Tyr Arg Ala
305                 310                 315                 320

Asp Leu Asp Pro Leu Asp Met Pro Cys Thr Thr Ile Pro Ser Ala Pro
                325                 330                 335

Gln Ala Val Ile Ser Ser Val Asn Glu Thr Ser Leu Met Leu Glu Trp
            340                 345                 350

Thr Pro Pro Arg Asp Ser Gly Gly Arg Glu Asp Leu Val Tyr Asn Ile
        355                 360                 365

Ile Cys Lys Ser Cys Gly Ser Gly Arg Gly Ala Cys Thr Arg Cys Gly
    370                 375                 380

Asp Asn Val Gln Tyr Ala Pro Arg Gln Leu Gly Leu Thr Glu Pro Arg
385                 390                 395                 400

Ile Tyr Ile Ser Asp Leu Leu Ala His Thr Gln Tyr Thr Phe Glu Ile
                405                 410                 415

Gln Ala Val Asn Gly Val Thr Asp Gln Ser Pro Phe Ser Pro Gln Phe
            420                 425                 430

Ala Ser Val Asn Ile Thr Thr Asn Gln Ala Ala Pro Ser Ala Val Ser
        435                 440                 445

Ile Met His Gln Val Ser Arg Thr Val Asp Ser Ile Thr Leu Ser Trp
    450                 455                 460

Ser Gln Pro Asp Gln Pro Asn Gly Val Ile Leu Asp Tyr Glu Leu Gln
465                 470                 475                 480

Tyr Tyr Glu Lys Glu Leu Ser Glu Tyr Asn Ala Thr Ala Ile Lys Ser
                485                 490                 495

Pro Thr Asn Thr Val Thr Val Gln Gly Leu Lys Ala Gly Ala Ile Tyr
            500                 505                 510

Val Phe Gln Val Arg Ala Arg Thr Val Ala Gly Tyr Gly Arg Tyr Ser
        515                 520                 525

Gly Lys Met Tyr Phe Gln Thr Met Thr Glu Ala Glu Tyr Gln Thr Ser
    530                 535                 540

Ile Lys Glu Lys Leu Pro Leu Ile Val Gly Ser Ser Ala Ala Gly Leu
545                 550                 555                 560

Val Phe Leu Ile Ala Val Val Val Ile Ala Ile Val Cys Asn Arg Arg
                565                 570                 575

Gly Phe Glu Arg Ala Asp Ser Glu Tyr Thr Asp Lys Leu Gln His Tyr
            580                 585                 590

Thr Ser Gly His Met Thr Pro Gly Met Lys Ile Tyr Ile Asp Pro Phe
        595                 600                 605

Thr Tyr Glu Asp Pro Asn Glu Ala Val Arg Glu Phe Ala Lys Glu Ile
    610                 615                 620

Asp Ile Ser Cys Val Lys Ile Glu Gln Val Ile Gly Ala Gly Glu Phe
625                 630                 635                 640

Gly Glu Val Cys Ser Gly His Leu Lys Leu Pro Gly Lys Arg Glu Ile
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645 650 655

Phe Val Ala Ile Lys Thr Leu Lys Ser Gly Tyr Thr Glu Lys Gln Arg
660 665 670

Arg Asp Phe Leu Ser Glu Ala Ser Ile Met Gly Gln Phe Asp His Pro
675 680 685

Asn Val Ile His Leu Glu Gly Val Val Thr Lys Ser Thr Pro Val Met
690 695 700

Ile Ile Thr Glu Phe Met Glu Asn Gly Ser Leu Asp Ser Phe Leu Arg
705 710 715 720

Gln Asn Asp Gly Gln Phe Thr Val Ile Gln Leu Val Gly Met Leu Arg
725 730 735

Gly Ile Ala Ala Gly Met Lys Tyr Leu Ala Asp Met Asn Tyr Val His
740 745 750

Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Asn Ser Asn Leu Val Cys
755 760 765

Lys Val Ser Asp Phe Gly Leu Ser Arg Phe Leu Glu Asp Asp Thr Ser
770 775 780

Asp Pro Thr Tyr Thr Ser Ala Leu Gly Gly Lys Ile Pro Ile Arg Trp
785 790 795 800

Thr Ala Pro Glu Ala Ile Gln Tyr Arg Lys Phe Thr Ser Ala Ser Asp
805 810 815

Val Trp Ser Tyr Gly Ile Val Met Trp Glu Val Met Ser Tyr Gly Glu
820 825 830

Arg Pro Tyr Trp Asp Met Thr Asn Gln Asp Val Ile Asn Ala Ile Glu
835 840 845

Gln Asp Tyr Arg Leu Pro Pro Pro Met Asp Cys Pro Ser Ala Leu His
850 855 860

Gln Leu Met Leu Asp Cys Trp Gln Lys Asp Arg Asn His Arg Pro Lys
865 870 875 880

Phe Gly Gln Ile Val Asn Thr Leu Asp Lys Met Ile Arg Asn Pro Asn
885 890 895

Ser Leu Lys Ala Met Ala Pro Leu Ser Ser Gly Ile Asn Leu Pro Leu
900 905 910

Leu Asp Arg Thr Ile Pro Asp Tyr Thr Ser Phe Asn Thr Val Asp Glu
915 920 925

Trp Leu Glu Ala Ile Lys Met Gly Gln Tyr Lys Glu Ser Phe Ala Asn
930 935 940

Ala Gly Phe Thr Ser Phe Asp Val Val Ser Gln Met Met Met Glu Asp
945 950 955 960

Ile Leu Arg Val Gly Val Thr Leu Ala Gly His Gln Lys Lys Ile Leu
965 970 975

Asn Ser Ile Gln Val Met Arg Ala Gln Met Asn Gln Ile Gln Ser Val
980 985 990

Glu Val

WE CLAIM:

1. A method of activating a ligand regulatory pathway in a cell, comprising reacting an Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein with a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the cell surface thereby activating the ligand regulatory pathway in the cell.

2. A method as claimed in claim 1 wherein the protein or part of the protein is lacking in catalytic kinase activity.

3. A method as claimed in claim 1 wherein the part of the protein comprises an extracellular domain of an Eph subfamily receptor tyrosine kinase.

4. A method for identifying a substance which is capable of binding to a ligand for an Eph subfamily receptor tyrosine kinase and activating a ligand regulatory pathway in a cell, comprising reacting a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase with at least one substance which potentially can bind with the ligand, under conditions which permit the formation of substance-ligand complexes, and assaying for substance-ligand complexes, for free substance, for non-complexed ligands, or for activation of the ligand.

5. A method as claimed in claim 4 wherein activation of the ligand is assayed by measuring phosphorylation of the ligand or binding of SH2 domains to the ligand, or by assaying for a biological affect on the cell.

6. A method as claimed in claim 4 wherein the substance is an Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein.

7. A method as claimed in claim 4 wherein the part of the protein comprises an extracellular domain.

8. A method as claimed in claim 4 wherein the biological affect on the cell is inhibition or stimulation of proliferation, differentiation or migration.

9. A method for assaying a medium for an agonist or antagonist of a ligand regulatory pathway in a cell which comprises providing a cell expressing a ligand for an Eph subfamily receptor tyrosine kinase on the cell surface, reacting the cell with an Eph subfamily receptor

tyrosine kinase protein and a suspected agonist or antagonist under conditions which permit the formation of ligand-receptor tyrosine kinase protein complexes on the cell surface, and assaying for ligand-receptor tyrosine kinase protein complexes, for free receptor tyrosine kinase protein, for non-complexed proteins, for activation of the receptor tyrosine kinase protein, or for activation of the ligand.

10. A method as claimed in claim 9 wherein activation of the ligand is assayed by measuring phosphorylation of the ligand or binding of SH2 domains to the ligand or by assaying for a biological affect on the cell.

11. A method as claimed in claim 9 wherein the biological affect on the cell is inhibition or stimulation of proliferation, differentiation or migration.

12. A method for affecting neuronal development or regeneration in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein.

13. A method as claimed in claim 12 wherein the part of the protein comprises an extracellular domain of an Eph subfamily receptor tyrosine kinase.

14. A method as claimed in claim 12 wherein the protein or part of the protein is lacking in a catalytic kinase domain.

15. A method for stimulating or inhibiting axonogenesis in a mammal comprising administering to a mammal an effective amount of a purified and isolated Eph subfamily receptor tyrosine kinase protein, or an isoform or a part of the protein having at least 20 contiguous amino acids of the protein.

16. A method as claimed in claim 15 wherein the part of the protein comprises about an extracellular domain of an Eph subfamily receptor tyrosine kinase.

17. A method as claimed in claim 15 wherein the protein or part of the protein is lacking in a catalytic kinase domain.

18. A pharmaceutical composition which comprises a purified and isolated Eph subfamily receptor tyrosine kinase protein or an isoform or a part of the protein having at

least 20 contiguous amino acids of the protein for affecting neuronal development or regeneration and a pharmaceutically acceptable carrier, diluent or excipient.

19. A pharmaceutical composition as claimed in claim 18 wherein the part of the protein comprises an extracellular domain of an Eph subfamily receptor tyrosine kinase.

20. A pharmaceutical composition as claimed in claim 18 wherein the protein or part of the protein is lacking in a catalytic kinase domain.

FIGURE 1

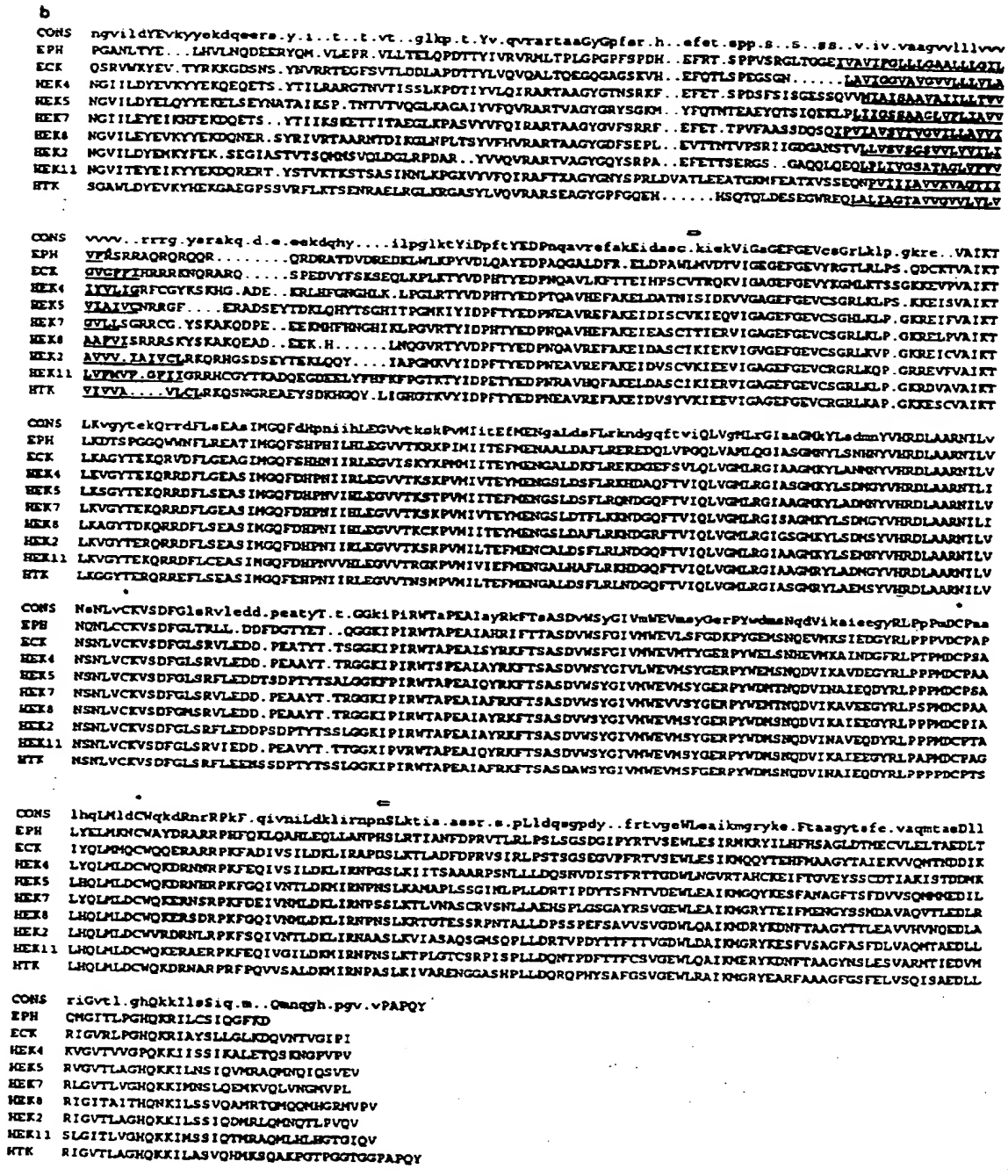

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FIGURE 2

```
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CTGGGCTGGATGGTACATCCCCCATCAGGGTGGGAAGAGGTGAGCGGCTACGACGAGAAC     180
ATGAACACTATCCGTACCTACCAGGTGTGCAATGTCTTTGAGTCAAGCCAGAACAACTGG     240
CTGCGGACCAAATTCATCCGGCGCCGTGGCGCCCACCGTATCCACGTGGAGATGAAGTTC     300
TCGGTGCGTGACTGCAGCAGCATTCCCAGCGTGCCGGGCTCCTGCAAGGAGACCTTCAAC     360
CTCTACTACTATGAGGCTGATTTTGACTTAGCCACCAAAACCTTTCCCAACTGGATGGAG     420
AATCCGTGGGTGAAGGTGGACACCATCGCGGCCGATGAGAGCTTCTCTCAGGTGGACCTG     480
GGTGGCCGCGTCATGAAAATCAACACTGAGGTGCGAAGCTTCGGTCCTGTGTCCCGCAAT     540
GGTTTCTACCTGGCCTTCCAGGACTACGGCGGCTGTATGTCCCTCATTGCTGTGCGCGTC     600
TTCTACCGGAAGTGCCCCCGAATCATCCAGAATGGTGCCATCTTCCAGGAGACACTATCG     660
GGGGCTGAGAGCACTTCGCTGGTGGCAGCTCGGGGCAGCTGCATCGCCAATGCTGAAGAA     720
GTGGACGTGCCCATCAAACTCTACTGTAACGGGGACGGCGAATGGCTGGTGCCCATCGGT     780
CGCTGCATGTGCAAGGCGGGCTTCGAGGCTGTGGAGAACGGCACCGTCTGCCGAGGTTGT     840
CCATCAGGAACCTTCAAGGCCAACCAAGGGGACGAAGCCTGCACCCACTGTCCCATCAAC     900
AGCCGCACCACCTCTGAGGGTGCCACCAACTGTGTATGCCGCAACGGCTACTACAGGGCC     960
GACCTGGACCCCTTAGACATGCCTTGCACAACCATCCCCTCTGCGCCCCAGGCTGTGATC    1020
TCCAGCGTCAACGAGACATCCCTCATGCTAGAGTGGACCCCACCCCGAGACTCCGGGGGT    1080
CGCGAGGATCTTGTTTACAACATCATCTGCAAGAGCTGTGGCTCCGGCCGGGGCGCATGC    1140
ACGCGCTGCGGGGACAACGTGCAGTACGCGCCCCGCCAGCTGGGCCTGACTGAGCCGCGC    1200
ATCTACATCAGTGACCTGCTGGCACACACGCAGTACACCTTCGAGATCCAGGCCGTGAAT    1260
GGTGTGACCGACCAGAGTCCCTTCTCACCTCAGTTCGCCTCTGTGAACATCACCACCAAC    1320
CAAGCAGCACCATCGGCCGTGTCCATCATGCACCAGGTGAGCCGCACTGTGGACAGCATC    1380
ACCCTGTCGTGGTCCCAGCCAGACCAGCCCAACGGTGTGATCCTGGACTACGAGCTGCAG    1440
TACTATGAGAAGGAGCTCAGTGAGTACAACGCCACGGCCATAAAAAGCCCCACCAACACA    1500
GTCACTGTGCAGGGCCTCAAAGCCGGCGCCATCTATGTCTTCCAGGTGCGGGCACGCACC    1560
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FIGURE 2 (cont'd)

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TACCAGACCAGCATCAAGGAAAAGCTACCCCTCATCGTTGGCTCCTCCGCCGCCGGCTTA 1680

GTCTTCCTCATCGCTGTGGTCGTCATTGCCATCGTATGTAACAGACGGGGGTTTGAGCGT 1740

GCCGACTCAGAGTACACGGACAAGCTACAGCACTACACCAGCGGACACATGACCCCAGGC 1800

ATGAAGATCTATATAGATCCTTTCACCTATGAAGATCCTAATGAGGCAGTGCGGGAGTTT 1860

GCCAAGGAAATTGACATCTCCTGTGTCAAGATTGAGCAGGTGATTGGAGCAGGGGAATTT 1920

GGTGAGGTCTGCAGTGGCCATTTGAAGCTGCCAGGCAAGAGAGAGATCTTTGTAGCCATC 1980

AAGACCCTCAAGTCAGGATACACGGAGAAACAGCGCCGGGACTTCCTGAGTGAGGCATCC 2040

ATCATGGGCCAGTTCGACCACCCCAATGTCATCCATCTGGAAGGGGTTGTCACCAAGAGC 2100

ACACCTGTCATGATCATCACTGAATTCATGGAGAATGGATCTCTGGACTCCTTCCTCCGG 2160

CAAAATGATGGGCAGTTCACAGTCATCCAACTGGTGGGCATGCTGAGGGGCATTGCAGCC 2220

GGCATGAAGTACCTGGCGGACATGAACTACGTGCACCGTGACCTTGCTGCTCGAAACATC 2280

CTCGTCAACAGTAACCTGGTGTGTAAGGTGTCTGACTTTGGGCTCTCACGCTTCCTGGAG 2340

GATGACACGTCTGAGCCCACCTATACCAGCGCTCTGGGTGGGAAGATCCCCATCCGTTGG 2400

ACGGCACCGGAAGCCATCCAGTACCGGAAATTCACCTCGGCCAGTGATGTGTGGAGCTAT 2460

GGCATCGTCATGTGGGAGGTGATGTCCTACGGGGAACGACCCTACTGGGACATGACCAAT 2520

CAAGACGTAATCAACGCCATTGAACAGGACTACAGACTACCTCCGCCCATGGACTGCCCT 2580

AGCGCCCTGCACCAGCTCATGCTGGACTGCTGGCAGAAGGACCGCAACCACCGGCCCAAG 2640

TTCGGCCAGATTGTCAACACGCTGGACAAGATGATCCGAAACCCCAACAGCCTCAAAGCC 2700

ATGGCACCCCTGTCCTCTGGCATCAACCTGCCACTGCTGGACCGCACGATACCGGACTAC 2760

ACCAGCTTTAACACAGTGGATGAGTGGCTAGAGGCCATCAAGATGGGCCAGTACAAGGAG 2820

AGCTTTGCCAACGCCGGCTTCACCTCTTTCGACGTTGTATCTCAGATGATGATGGAGGAC 2880

ATTCTCCGCGTTGGGGTCACTCTAGCTGGCCACCAGAAAAAAATCCTGAACAGTATCCAG 2940

GTGATGCGGGCCCAGATGAACCAGATCCAGTCTGTAGAGGTTTGACATTCGCCTGCCTCG 3000

GTTCTCCTCTTCCTCCACGCCGCCCCTGAGCCCCTACGTCGGTCCCTGCTGCTCTGTCAC 3060

TGCAGGTCAGCACTGCCAGGAGGCCACAGACAACAGGAAGACCAA 3105

FIGURE 3

```
MGARVPVLPGLDGSFCWLLLLPLLAAVEETLMDSTTATAELGWMVHPPSG
WEEVSGYDENMNTIRTYQVCNVFESSQNNWLRTKFIRRRGAHRIHVEMKF  100
SVRDCSSIPSVPGSCKETFNLYYYEADFDLATKTFPNWMENPWVKVDTIA
ADESFSQVDLGGRVMKINTEVRSFGPVSRNGFYLAFQDYGGCMSLIAVRV  200
FYRKCPRIIQNGAIFQETLSGAESTSLVAARGSCIANAEEVDVPIKLYCN
GDGEWLVPIGRCMCKAGFEAVENGTVCRGCPSGTFKANQGDEACTHCPIN  300
SRTTSEGATNCVCRNGYYRADLDPLDMPCTTIPSAPQAVISSVNETSLML
EWTPPRDSGGREDLVYNIICKSCGSGRGACTRCGDNVQYAPRQLGLTEPR  400
IYISDLLAHTQYTFEIQAVNGVTDQSPFSPQFASVNITTNQAAPSAVSIM
HQVSRTVDSITLSWSQPDQPNGVILDYELQYYEKELSEYNATAIKSPTNT  500
VTVQGLKAGAIYVFQVRARTVAGYGRYSGKMYFQTMTEAEYQTSIKEKLP
LIVGSSAAGLVFLIAVVVIAIVCNRRGFERADSEYTDKLQHYTSGHMTPG  600
MKIYIDPFTYEDPNEAVREFAKEIDISCVKIEQVIGAGEFGEVCSGHLKL
PGKREIFVAIKTLKSGYTEKQRRDFLSEASIMGQFDHPNVIHLEGVVTKS  700
TPVMIITEFMENGSLDSFLRQNDGQFTVIQLVGMLRGIAAGMKYLADMNY
VHRDLAARNILVNSNLVCKVSDFGLSRFLEDDTSDPTYTSALGGKIPIRW  800
TAPEAIQYRKFTSASDVWSYGIVMWEVMSYGERPYWDMTNQDVINAIEQD
YRLPPPMDCPSALHQLMLDCWQKDRNHRPKFGQIVNTLDKMIRNPNSLKA  900
MAPLSSGINLPLLDRTIPDYTSFNTVDEWLEAIKMGQYKESFANAGFTSF
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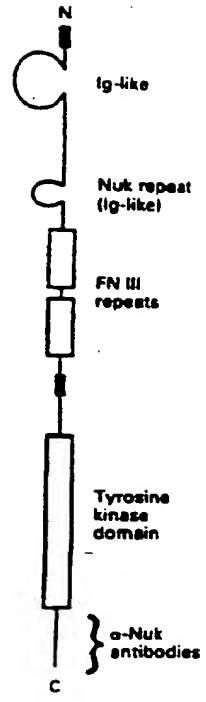

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FIGURE 4

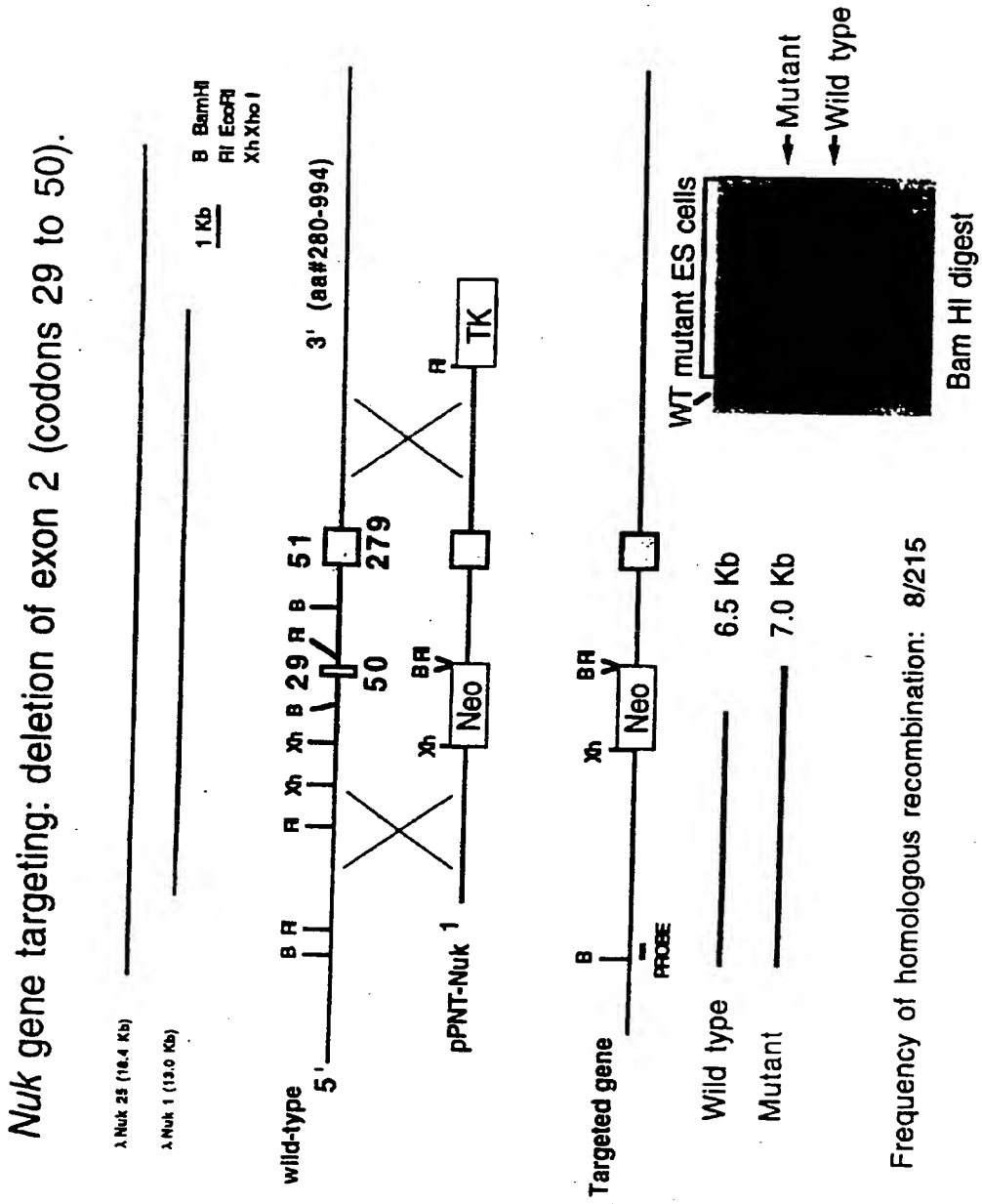

Nuk gene targeting: deletion of exon 2 (codons 29 to 50).
λ Nuk 25 (18.4 Kb)
λ Nuk 1 (13.0 Kb)
1 Kb
B BamHI
RI EcoRI
Xh Xho I
wild-type
5'
B R
R Xh
Xh B
B 29 R B
51
3' (aa#280-994)
279
50
pPNT-Nuk 1
BR
Xh
Neo
R
TK
Targeted gene
B
PROBE
Wild type
6.5 Kb
Mutant
7.0 Kb
WT mutant ES cells
Mutant
Wild type
Bam HI digest
Frequency of homologous recombination: 8/215

FIGURE 5

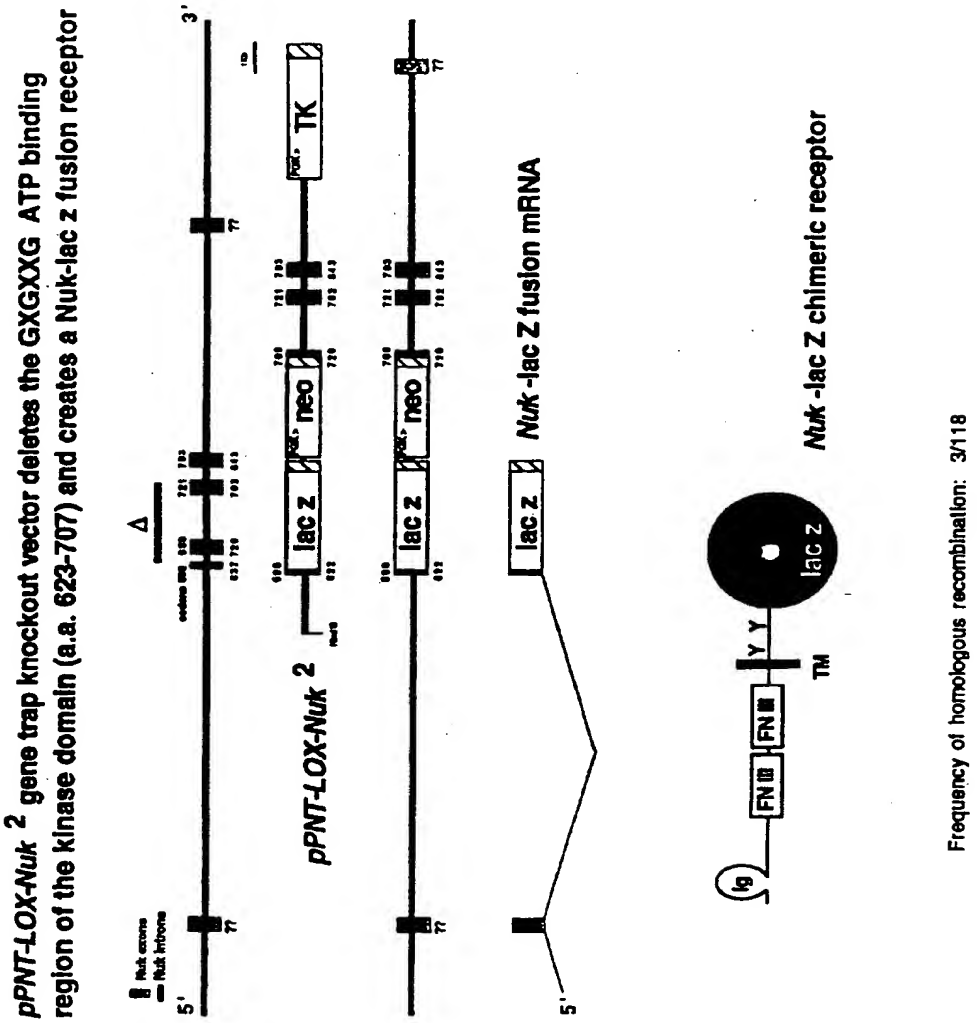

pPNT-LOX-Nuk 2 gene trap knockout vector deletes the GXGXXG ATP binding region of the kinase domain (a.a. 623-707) and creates a Nuk-lac z fusion receptor
Δ
lac z
neo
TK
pPNT-LOX-Nuk 2
Nuk -lac Z fusion mRNA
Nuk -lac Z chimeric receptor
FN III
TM
Frequency of homologous recombination: 3/118

FIGURE 6A

FIGURE 6B

FIGURE 6C

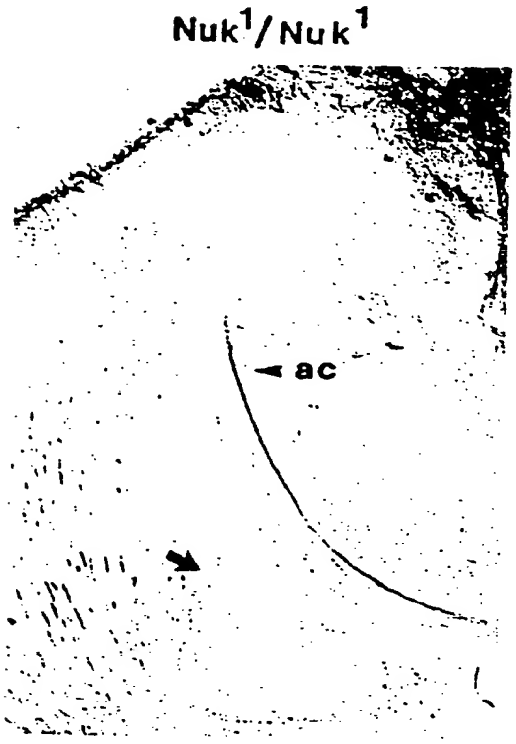


FIGURE 6D

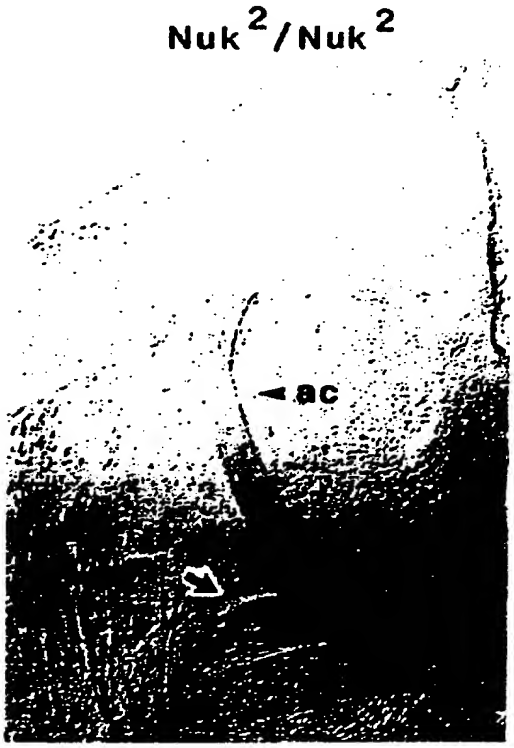


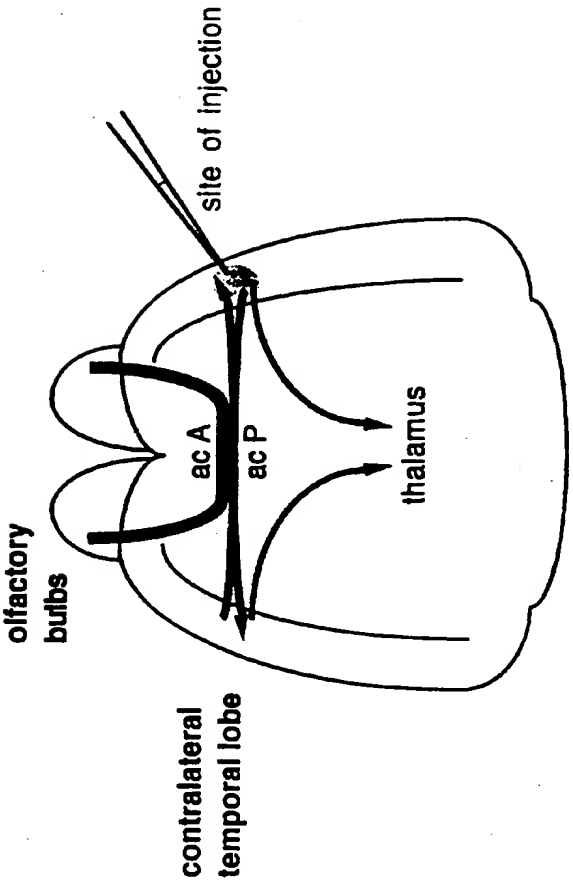
FIGURE 7A

FIGURE 7B

FIGURE 8

FIGURE 9

Fast Blue dye tracing of the temporal lobe



In normal mice and Nuk2/Nuk2 homozygotes the dye traces to the contralateral temporal lobe and to the thalamus.
In Nuk1/Nuk1 homozygotes, the dye falls to trace into the contralateral lobe. The dye does trace, however, to the thalamus indicating that this axon pathway is not affected.

FIGURE 10

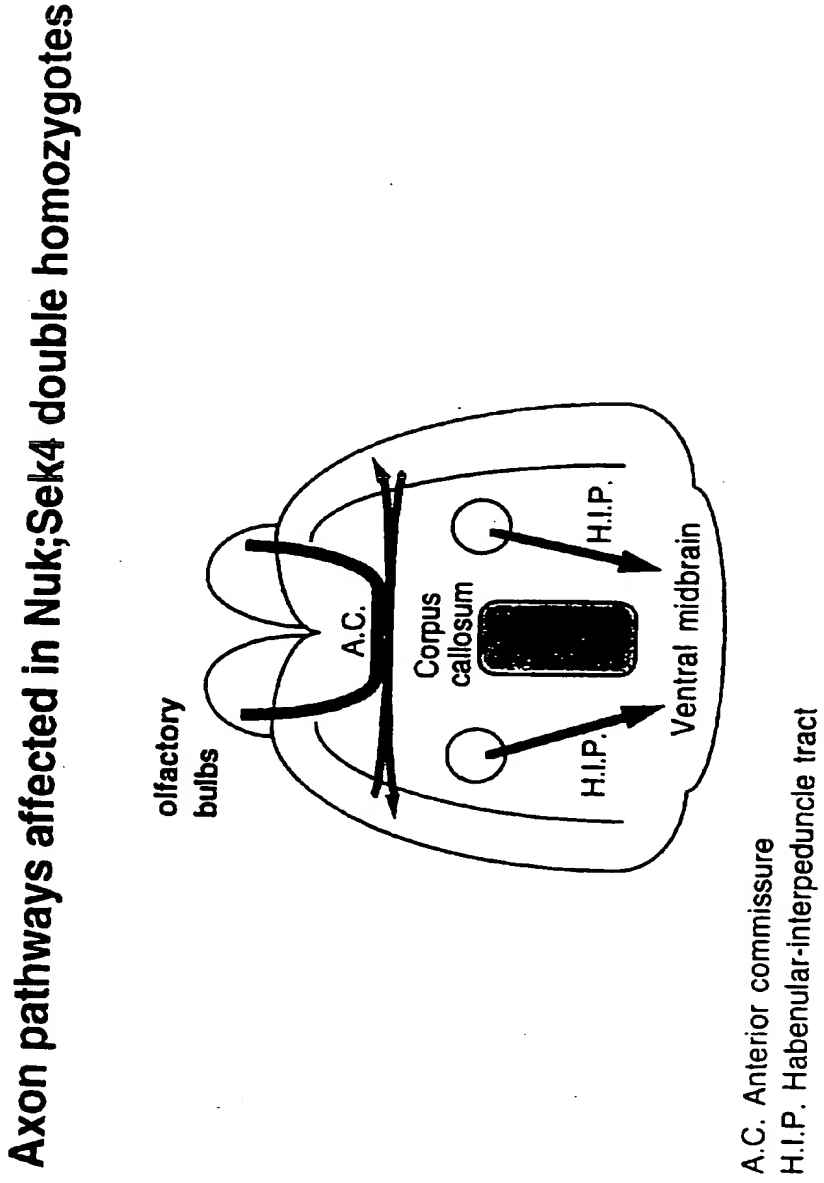

Axon pathways affected in Nuk;Sek4 double homozygotes
olfactory bulbs
A.C.
Corpus callosum
H.I.P.
H.I.P.
Ventral midbrain
A.C. Anterior commissure
H.I.P. Habenular-interpeduncle tract

FIGURE 11

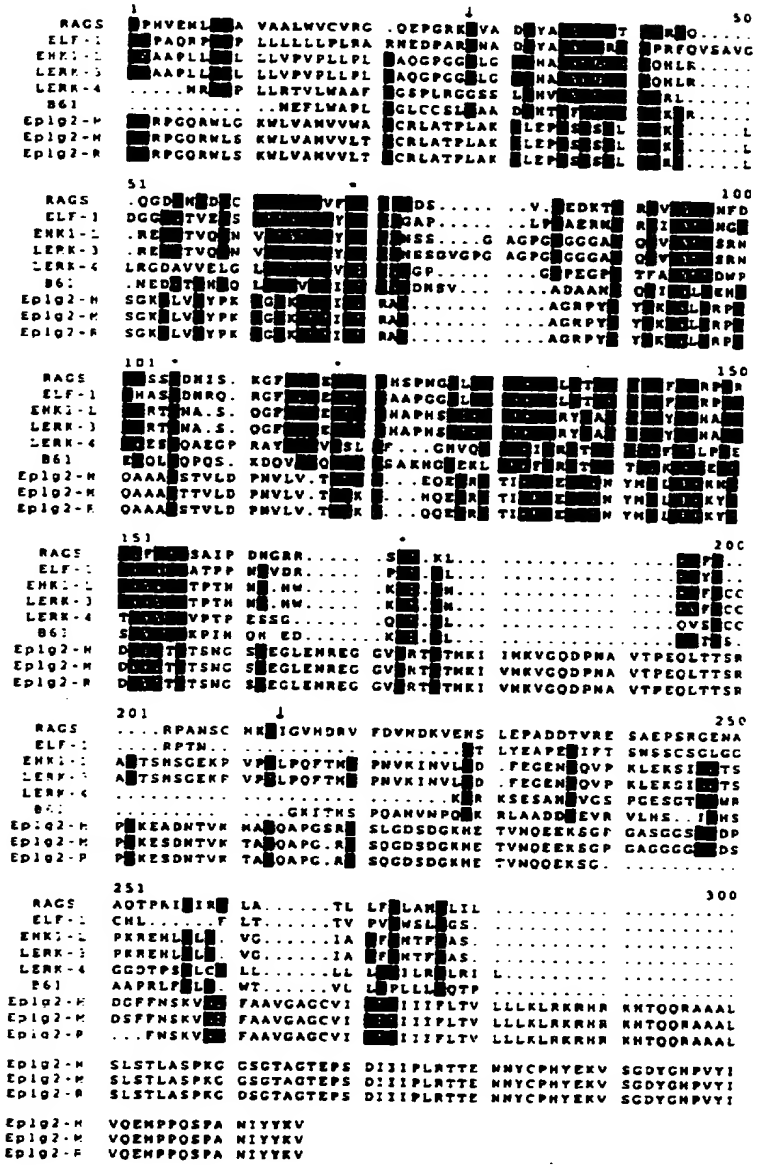


FIGURE 12

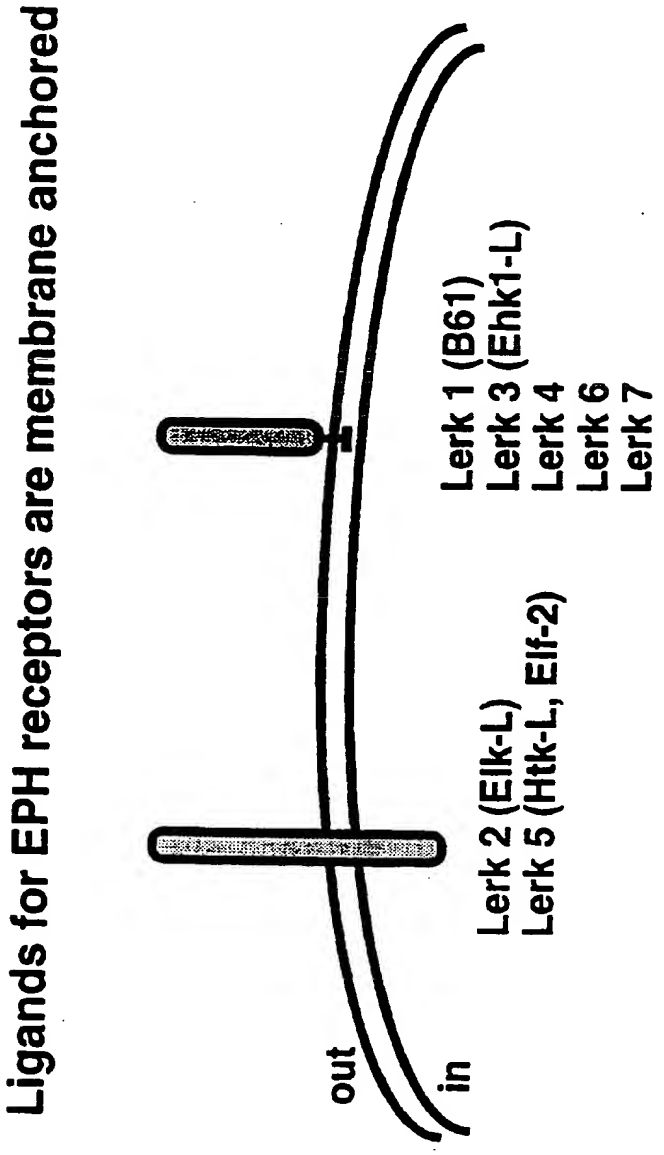
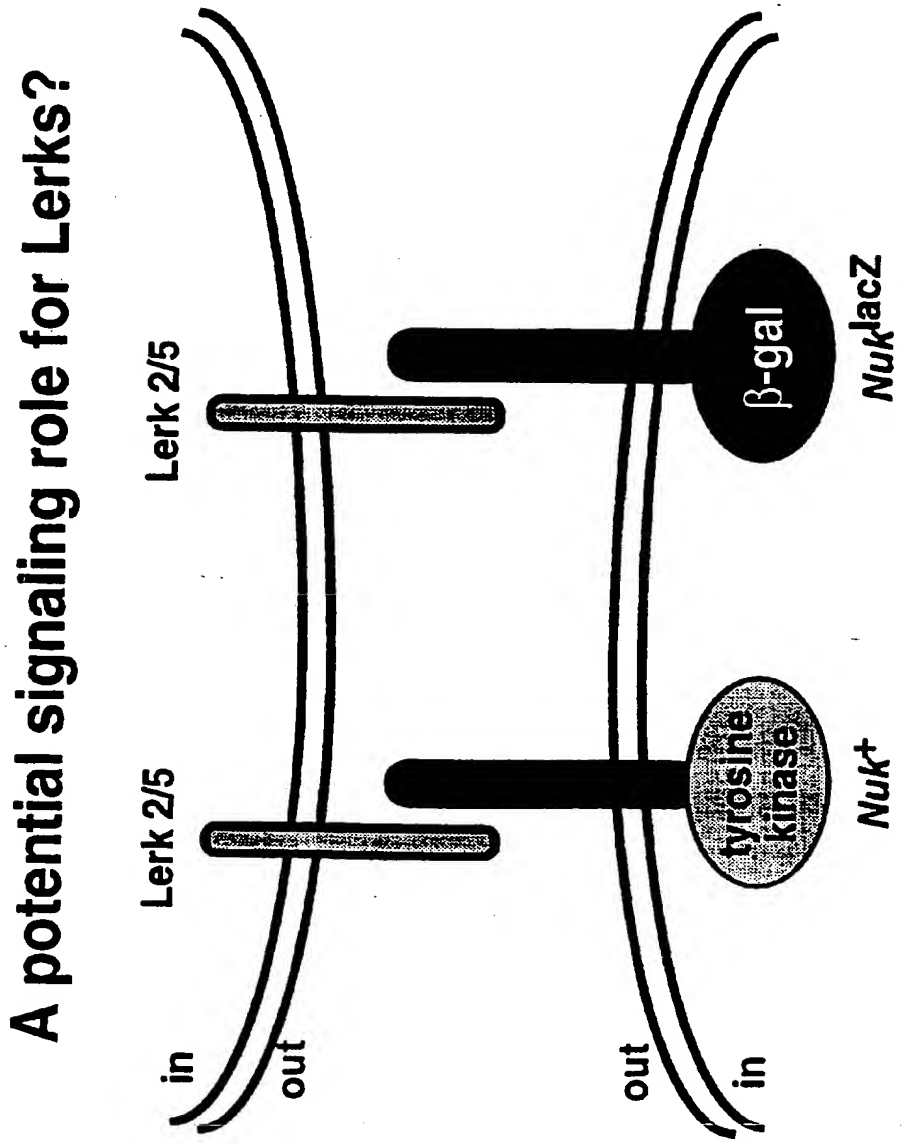

Ligands for EPH receptors are membrane anchored
Lerk 1 (B61)
Lerk 3 (Ehk1-L)
Lerk 4
Lerk 6
Lerk 7
Lerk 2 (Elk-L)
Lerk 5 (Htk-L, Elf-2)
out
in

FIGURE 13


A potential signaling role for Lerks?
Lerk 2/5
Lerk 2/5
β-gal
Nuk lacZ
tyrosine kinase
Nuk+
in
out
out
in

INTERNATIONAL SEARCH REPORT

International Application No
PL , CA 96/00679

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C12N9/12 A61K38/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 14776 A (GENENTECH INC ;NEW ENGLAND DEACONESS HOSPITAL (US)) 1 June 1995 see claim 10	18
X	WO 95 27060 A (REGENERON PHARMA) 12 October 1995 see claim 8	18
A	WO 93 00425 A (INST MEDICAL W & E HALL) 7 January 1993 see page 9 - page 11	4-11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 1997	26 -02- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoekstra, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
P../CA 96/00679

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURR. BIOL. (1995), 5(2), 168-78 CODEN: CUBLE2;ISSN: 0960-9822, 1995, XP002024805 VETTER, MONICA L. ET AL: ".beta. PDGF receptor mutants defective for mitogenesis promote neurite outgrowth in PC12 cells" see the whole document	1-20
A	PROC. NATL. ACAD. SCI. U. S. A. (1993), 90(12), 5404-8 CODEN: PNASA6;ISSN: 0027-8424, 1993, XP002024806 TOYOSHIMA, HIDEO ET AL: "Differently spliced cDNAs of human leukocyte tyrosine kinase receptor tyrosine kinase predict receptor proteins with and without a tyrosine kinase domain and a soluble receptor protein" see the whole document	1-20
P,X	WO 95 28484 A (AMGEN INC) 26 October 1995 see claim 30 see page 12	1,12,18
P,X	WO 95 30326 A (MOUNT SINAI HOSPITAL CORP ;PAWSON ANTHONY (CA); HENKEMEYER MARK (C) 9 November 1995 see page 8, line 15 - page 9, line 19; claims 9,12,13	4-11
P,X	WO 96 26958 A (HARVARD COLLEGE) 6 September 1996 see claim 46	1,12-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 96/00679

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-3 and 12-17 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC./CA 96/00679

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9514776	01-06-95	AU-A-	1180095	13-06-95
		AU-A-	1210895	13-06-95
		CA-A-	2175892	01-06-95
		CA-A-	2175893	01-06-95
		EP-A-	0730646	11-09-96
		EP-A-	0730740	11-09-96
		WO-A-	9514930	01-06-95
WO-A-9527060	12-10-95	AU-A-	2278995	23-10-95
		CA-A-	2187167	12-10-95
		ZA-A-	9502762	20-02-96
WO-A-9300425	07-01-93	AU-B-	655299	15-12-94
		EP-A-	0590030	06-04-94
		JP-T-	6508747	06-10-94
		NZ-A-	243252	27-11-95
WO-A-9528484	26-10-95	AU-A-	2292595	10-11-95
		CA-A-	2189028	26-10-95
		EP-A-	0756627	05-02-97
WO-A-9530326	09-11-95	CA-A-	2122874	30-10-95
		CA-A-	2186365	09-11-95
WO-A-9626958	06-09-96	NONE		

Form PCT/ISA/210 (patent family annex) (July 1992)